Studies of ion transport *in vivo* in the spontaneously hypertensive rat; Implications for essential hypertension

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To my wife Sarah
and my children
Hannah and William

CORRIGENDA

p. 24 second paragraph. This should read: When the B_1 field is uniform the signal received by the coil from the sample lying in the uniform part of the B_1 field, immediately after a 90^0 pulse, is proportional to both the number of nuclei in the sample and the sample volume. This can be expressed by the following equation:

p. 206. The reference Helpern et al., 1987, is not printed in the correct alphabetical order and should be printed after the reference Heisler and Piiper 1972.

CONTENTS

DECLARATION

The work described in this thesis was carried out in the departments of Clinical pharmacology and the MRC Biochemical and Clinical Magnetic Resonance Unit at the University of Oxford between February 1987 and August 1990 while I held a MRC Clinical Scientists post.

I prepared this dissertation alone. I designed all of the experiments presented in this thesis. I built the radiofrequency coil that was used to measure absolute rubidium concentrations. I performed most of the NMR spectroscopy, analysed all the data and carried out most of the laboratory work, including measuring rubidium concentrations using atomic absorption spetrophotometry. The blood and muscle lactate concentrations were measured by Mrs Yvonne Green.

Some of the material in this dissertation has already, or will shortly be published elsewhere. Permission has been obtained for reproduction of material in this dissertation that has been published elsewhere. A list of publications will follow.

PUBLICATIONS

Syme PD, Allis JL, Dixon RM, Aronson JK, Grahame-Smith DG, Radda GK. Quantification of rubidium concentrations in rat muscle *in vivo* using high resolution nuclear magnetic resonance spectroscopy. *Abstr 7th Annu Mg Soc Magn Reson Med* 1988;924.

Syme PD, Allis JL, Dixon RM, Aronson JK, Grahame-Smith DG, Radda GK. A non-invasive method of measuring concentrations of rubidium *in vivo* in rat skeletal muscle by ^{87}rubidium NMR spectroscopy; Implications for the *in vivo* measurement of cation transport activity. *Clin Sci* 1990; **78:** 303-9

Syme PD, Dixon RM, Aronson JK, Grahame-Smith DG, Radda GK. Evidence for increased *in vivo* Na^+/K^+-ATPase activity in skeletal muscle of spontaneously hypertensive rats. *Clin Sci* 1990; **78:** 8p.

Syme PD, Arnolda L, Aronson JK, Grahame-Smith DG, Radda GK. Evidence for increased *in vivo* Na^+/H^+ antiporter activity in skeletal muscle of spontaneously hypertensive rats.*Clin Sci* 1990; **78:** 8p.

Syme PD, Arnolda L, Green Y, Aronson JK, Grahame-Smith DG, Radda GK. Evidence for increased *in vivo* Na^+/H^+ antiporter activity and an altered skeletal muscle contractile response in the spontaneously hypertensive rat. *J Hypertens* 1990; 8: 1027-36.

Syme PD, Dixon RM, Aronson JK, Grahame-Smith DG, Radda GK. Evidence for increased *in vivo* sodium/potassium pump activity and potassium efflux in skeletal muscle of spontaneously hypertensive rats. *J Hypertens* 1990; **8:** 1161-6.

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Syme PD, Green Y, Aronson JK, Radda GK. Reversal by nifedipine of increased in vivo Na^+/H^+ antiporter activity in the skeletal muscle of spontaneously hypertensive rats. *J Hypertens* 1990; **8:** S113.

Syme PD, Arnolda L, Aronson JK, Radda GK (1990) Increased Na^+/H^+ antiporter activity *in vivo* in skeletal muscle of spontaneously hypertensive rats can be reversed by nifedipine. *J Hypertens*; **8:** 1064-1065.

Syme PD, Green Y, Aronson JK, Radda GK. The effect of L-type calcium channel blockade on *in-vivo* Na^+/H^+ antiporter activity in the skeletal muscle of the spontaneously hypertensive rat 1991 (submitted for publication).

Syme PD, Brunotte F, Green Y, Aronson JK, Radda GK. The effect of β_2-adrenoreceptor stimulation and blockade of L-type calcium channels on *in vivo* Na^+/H^+ antiporter activity in skeletal muscle. 1991 (submitted for publication)

Syme PD, Aronson JK, Thompson CH, Williams EM, Green Y, Radda GK. The role of Na^+/H^+ antiporter activity, intrinsic buffering, and bicarbonate/chloride exchange in the control of pH in skeletal muscle of the spontaneously hypertensive rat *in vivo*. 1991 (submitted for publication).

Syme PD, Aronson JK. Ion transport abnormalities in essential hypertension: a hypothesis. 1991 (submitted for publication).

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Dr Jeffrey Aronson for his helpful advice and enjoyable discussion. Dr Jonathon Allis for teaching me the principles of radiofrequency coil building. Dr Ruth Dixon for teaching me how to use the NMR spectrometer. Mrs Yvonne Green for expert technical assistance.

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ABSTRACT

The work presented in this thesis involves new methods of using nuclear magnetic resonance spectroscopy (NMR) to study ion transport *in vivo* in hypertension.

A new NMR technique was developed which allowed measurement of absolute concentrations of rubidium (potassium) *in vivo*. This method was used to study rubidium (potassium) kinetics in spontaneously hypertensive rats (SHRs) and Wistar-Kyoto (WKY) rats and showed evidence for increased potassium efflux and increased Na^+/K^+-ATPase activity in SHR skeletal muscle *in vivo*.

NMR was also used to study *in vivo* Na^+/H^+ antiporter activity in the skeletal muscle of the rat and it was found that Na^+/H^+ antiporter activity is increased by β_2-adrenoceptor stimulation and by increasing the activity of the sympathetic nervous system. This stimulation of Na^+/H^+ antiporter activity in skeletal muscle by the sympathetic nervous system may be part of the well known but poorly understood "fright and flight" mechanism.

Using these new NMR techniques it was found that Na^+/H^+ antiporter activity is increased in SHR skeletal muscle *in vivo*. In addition, this difference in antiport activity can be removed by blocking L-type calcium channels.

These differences in ion transport in SHRs occured in association with increased skeletal muscle relaxation following contraction which is similar to the finding in vascular smooth muscle in essential hypertension.

Despite differences in *in vivo* Na^+/H^+ antiporter activity and *in vivo* potassium fluxes in SHRs no difference was found in either intracellular steady-state concentrations of potassium ions, hydrogen ions or bicarbonate. In addition, there was no difference in intrinsic cell buffering in SHRs *in vivo* compared with WKY rats.

On the basis of these findings a new hypothesis is proposed which links ion transport abnormalities found in hypertension with cell volume and pH regulation.

Chapter One

Introduction and Aims

In this chapter I will review some aspects of ion transport in cells. I will concentrate on control of ion transport in relation to cell volume and pH regulation which are relevant to much of the work that will be presented in the following chapters and I believe relevant to ion transport abnormalities found in hypertension. I will then give a brief outline of some of the common ion transport abnormalities found in genetic hypertension and the problems that have been reported in *in vitro* methods of studying ion transport. I will finish this chapter by outlining the main aims of this project

1.1 SOME ASPECTS OF CELL VOLUME AND pH REGULATION

1.11 Cell volume regulation

Regulation of cell volume is an essential requirement for every cell. As the cell membrane is freely permeable to water, the volume of the cell is determined by the number of osmotically active particles in the cell. In all cells intracellular ions rather than cellular proteins are responsible for the majority of the cell's osmotic potential (for a review see Sten -Knudsen, 1978) and, therefore, every cell has to be able to keep the concentration of these osmotically active ions constant. The two most important osmotically-active cations are sodium and potassium. Thus, control of the movement of these cations across the cell membrane is critical for cell volume regulation.

In contrast, calcium does not contribute directly to the cell osmotic potential since the majority of calcium in the cell is bound (Postnov and Orlov; 1985; Brostrom and Brostrom, 1990). The role of calcium in cell volume regulation is more complex. Calcium may act as a second messenger (Metcalfe et al., 1986; for a review see Bolton 1979), controlling the opening and activation of sodium and potassium membrane transporters which are important for volume regulation.

As there is a continuous tendency for the cell to loose potassium and to a lesser extent gain sodium the cell has devised ways of keeping the concentrations of these ions constant (Sten-Knudsen, 1978; Leaf, 1957). As the maintenance of a high intracellular potassium and a low intracellular sodium occurs against the concentration gradients for these ions, this is also an energy-dependent process. The membrane transporter responsible for these gradients is the Na^+-pump , Na^+/K^+-ATPase (E.C. 3.6.1.37), which transports 3 intracellular Na^+ ions for 2 extracellular K^+ions (Garrahan and Garay, 1974). This important membrane transporter is present in the membrane of nearly all eukaryotic cells and its activity is carefully regulated (for a review see Schwartz et al.,1975).

In addition to maintaining constant concentrations of sodium and potassium and a constant cell volume under resting conditions, cells have the ability to restore there volume when this is artificially altered *in vitro,* when they are placed in either a hypo- or hyper- osmolar external environment. The cell is very sensitive to changes in volume and mechanisms restoring volume back to normal can be triggered by volume changes of less than 5% (Hudson and Schultz, 1988). The cell

responds to a cell volume *decrease* by activating processes which increase intracellular sodium resulting in a regulatory cell volume increase (RVI), and to a cell volume *increase* by activating processes which reduce intracellular potassium resulting in a regulatory volume decrease (RVD)(for a review see Hoffman and Simonsen, 1989).

These volume regulatory mechanisms do not change the membrane potential because movements of ions across the cell membrane are balanced by the exchange of another ion, for example, the Na^+/H^+ antiport exchanges an external sodium ion for an intracellular proton, with no change in the membrane potential.

The ion transporters involved in cell regulatory volume increase are the Na^+/H^+antiport and the bicarbonate/chloride exchanger (Fig. 1a). A combination of increased Na^+/H^+ exchange and bicarbonate/chloride exchange results in an increase in the total concentration of cell Na^+ with a resultant increase in cell osmotic potential and so an increase in total cell water with cell swelling. A change in the intracellular pH is prevented by release of protons from intracellular buffers.

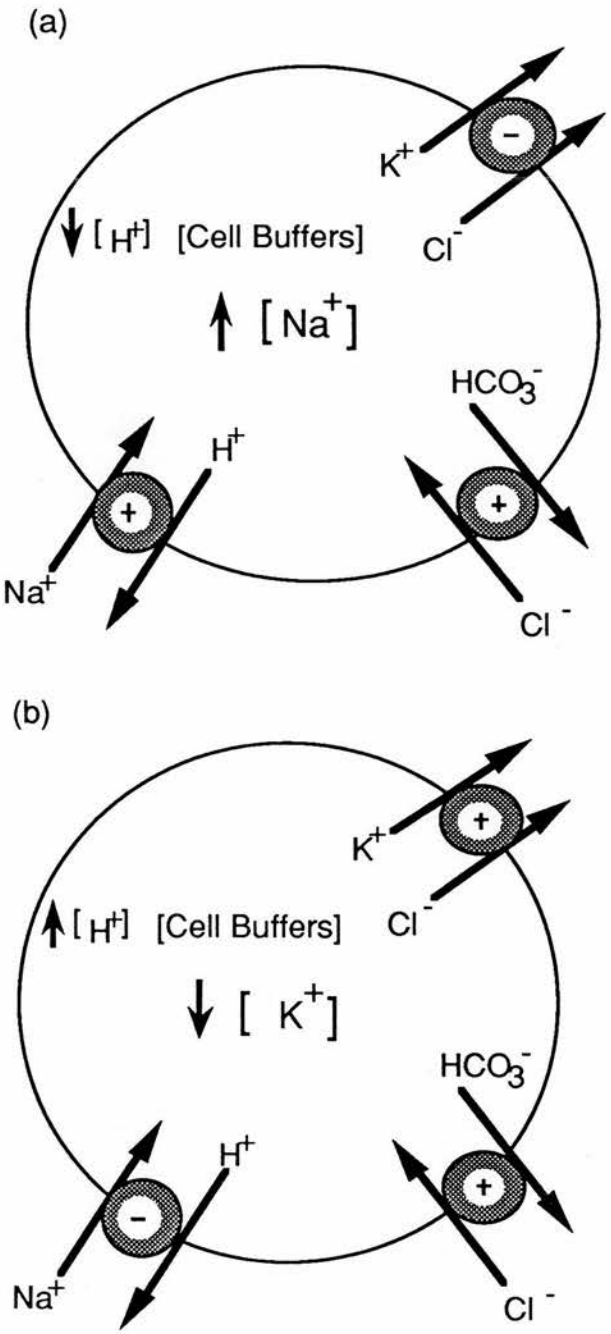


Fig.1.1 Mechanisms regulating cell volume resulting in (a) a regulatory cell volume increase and (b) a regulatory cell volume decrease.

The ion transporters involved in a regulatory volume decrease include potassium and chloride channels and also the bicarbonate/chloride exchanger (Fig. 1b). Increased opening of potassium and chloride channels with increased potassium and chloride efflux and increased bicarbonate/chloride exchange decreases intracellular potassium with a resultant decrease in cell osmotic potential and a cell volume reduction. The loss of potassium from the cell is greater than chloride because of bicarbonate/ chloride exchange and the resultant increase in hydrogen ion concentration is buffered by intracellular buffers.

The mechanism responsible for RVI is largely unknown but may involve a change to the cytoskeleton of the cell. However, there are many compounds that activate Na^+/H^+ exchange, including cell mitogens (for a review see Grinstein, 1986) and any of these substances have the potential for increasing cell volume. The mechanism responsible for a RVD is also unknown but calcium has been implicated in RVD at least in some cells (for reviews see Hoffman and Simonsen, 1989 and Schwartz and Passow, 1983).

The changes described above following a change in the osmolality of the extracellular fluid bathing the cell does not arise *in vivo* because the external environment of the cell is constant. However, the above mechanisms of regulating volume will be activated *in vivo* in situations where an external stimulus changes potassium or sodium fluxes across the cell membrane altering the osmotic potential of the cell (Hoffman and Simonsen, 1989). This will be discussed later in Chapter 10 in

relation to vascular smooth muscle cell stimulation to contraction with agonists such as angiotensin II or noradrenaline.

To conclude, cell volume regulatory mechanisms, in conjunction with Na^+/K^+-ATPase, are important *in vivo* for maintaining intracellular sodium and potassium ion concentrations following exposure to substances that have a tendency to change the intracellular concentrations of these ions and alter the osmotic potential of the cell.

1.12 Cell pH regulation

In the above section I briefly discussed the mechanisms by which cells regulate their volume. It is clear that these processes are also related to the control of pH in the cell.

In Figure 1.2 I have outlined some of the important mechanisms by which a cell regulates intracellular pH. Regulation of intracellular pH like regulation of cell volume is essential for cell survival. For a review of the cellular processes altered by a change in intracellular pH see Busa and Nuccetelli (1984).

These mechanisms maintain the intracellular pH of the cell about 1.5 pH units above that pH that would be determined by passive diffusion of hydrogen ions across the cell membrane (Roos and Boron, 1981).

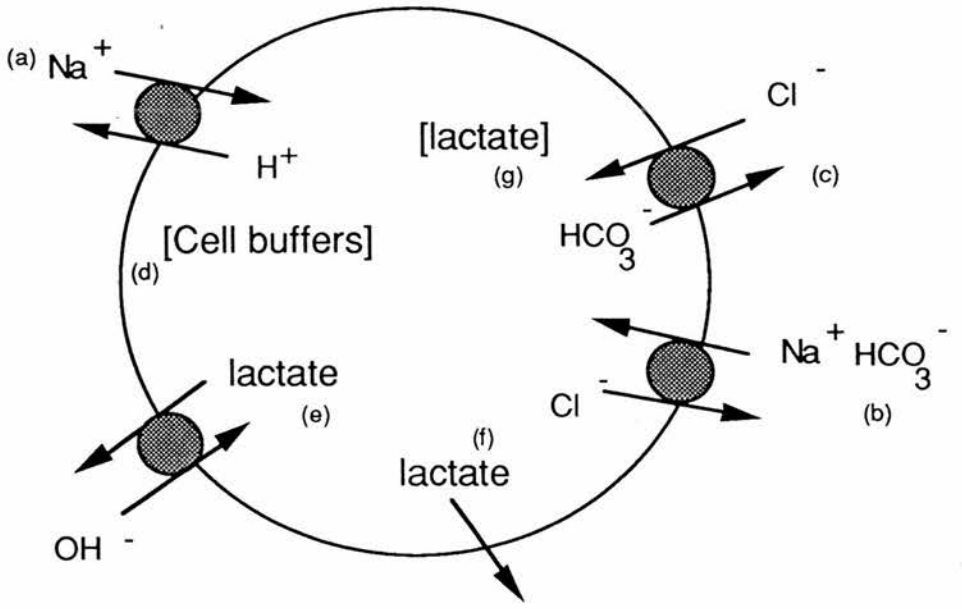


Fig. 1.2 Mechanisms involved in determining intracellular pH (a) Na^+/H^+ antiporter (b) sodium-dependent bicarbonate/chloride exchange (c) sodium-independent bicarbonate/chloride exchange (d) intracellular buffers eg. cellular proteins, inorganic phosphate and in muscle phosphocreatine. (e) lactate monocarboxylate carrier which exchanges one molecule of intracellular lactate for an extracellular hydroxyl ion. (f) passive lactate efflux (g) acid production represented by {lactate. At steady-state intracellular pH, acid production and acid influx will be balanced by the acid efflux mechanisms shown above (a-f). During contraction of muscle the most important mechanisms of acid efflux are Na^+/H^+ exchange and lactate efflux.

1.121 *Na^+/H^+ antiporter*

The Na^+/H^+ antiporter is one of the most important mechanisms for controlling intracellular pH, and it does this by exchanging extracellular Na^+ ions for intracellular H^+ ions with a stoichiometry of 1:1, with kinetics suggesting a single binding site for both sodium and hydrogen ions. (Grinstein and Rothstein, 1986). The Km for extracellular sodium is around 6 mmol/l and so at normal physiological concentrations of extracellular sodium the antiporter is saturated for sodium and so Na^+/H^+ exchange is regulated by intracellular hydrogen ion concentrations. There is also some evidence that intracellular protons also act as allosteric modifiers of the Na^+/H^+ antiport (Aronson et al., 1982). The Km for hydrogen ions varies depending on the cell investigated (Frelin et al., 1986) ranging from pH 6.3 to pH 7.4. In skeletal muscle the Km of the exchanger is 6.9×10^{-8} M, equivalent to a pH of 7.16 (Vigne et al., 1985). External H^+ ions can act as competitive (Aronson, 1985) and non-competitive inhibitors (Vigne et al., 1982) of external Na^+ ions, depending on the cell type, which in the latter case can alter antiporter activity within the physiological range of external pH .

The Na^+/H^+ antiport is reversibly inhibited by amiloride and amiloride derivatives (Frelin et al., 1987). The antiport can be activated by various substances including cell mitogens (for a review see Grinstein et al., 1989). This activation of the Na^+/H^+ antiporter is due to a shift in the Km of the antiporter by 0.2-0.3 pH units. At a molecular level the

antiport is activated by kinases including protein kinase C (for a review see Aronson and Boron, 1986).

Whether the antiporter controls pH or results in a regulatory volume increase, as discussed above, depends to some extent on the buffering capacity of the cell. In the hypothetical situation where the buffering capacity is infinite, the aniport will never reach equilibrium and will continue to transport Na^+ions into the cell and so will act entirely as a volume regulator. If on the other hand there is no buffering capacity then the antiporter will reach equilibrium very quickly and not change the total osmotic potential of the cell. *In vivo* the situation lies between these two extremes and so the antiporter acts as both a cell volume and a pH regulator

1.122 *Sodium-dependent and -independent bicarbonate/chloride exchange*

In skeletal and vascular smooth muscle the bicarbonate/chloride exchangers can result in both bicarbonate influx and efflux. In these tissues the operation of the sodium-independent bicarbonate/chloride exchanger results in a net chloride influx in exchange for intracellular bicarbonate, resulting in intracellular acidification. This occurs because the chemical concentration gradient for chloride predominates over that of bicarbonate. However, during acidification of the cell there is a reversal of the direction of bicarbonate flux with a sodium-dependent influx of bicarbonate in exchange for intracellular chloride, resulting in intracellular alkalinization (for a review see Cala and Grinstein,

1988). In experiments using ammonium chloride to load the skeletal muscle cell with acid, bicarbonate/chloride exchange accounts for about 20% (Aickin and Thomas, 1977a) of intracellular pH recovery. However, in acidosis resulting from increased skeletal muscle contraction the majority of pH recovery arises from lactate efflux of the cell and Na^+/H^+ antiport activity (Juel, 1988). Both sodium-dependent and sodium-independent bicarbonate exchange are inhibited by 4,4´-di-isothiocyanostilbene-2,2´-disulphonate (DIDS) (Aickin, 1986; Kahn et al., 1990)

1.123 *Lactate/Monocarboxylate exchanger*

Lactate efflux from skeletal muscle is another important mechanism for controlling pH during contraction (Juel, 1988). There are two mechanisms responsible for lactate efflux, passive diffusion of lactate down the lactate concentration gradient and a lactate exchange with an extracellular hydroxyl ion. This exchange is stimulated by intracellular acidosis and is inhibited by cinnamate. From *in vitro* data this exchanger would appear to be quantitatively as important as the Na^+/H^+ antiport in restoring pH during skeletal muscle contraction (Juel, 1988).

1.124 *Intrinsic buffering*

In addition, to the above mechanisms the cell has intracellular buffers which prevent changes in pH. These buffers include a variety of intracellular proteins and in the muscle cell, inorganic phosphate and phosphocreatine. Collectively buffering from these compounds is given

the term intrinsic intracellular buffering. Intrinsic buffering varies depending on the cell type but is of the order of 20-25 mmol of H^+ ions/pH unit for myocardium (Wolfe et al., 1988) and about 40-50 mmol H^+ ions/pH units for skeletal muscle (Aickin and Thomas, 1977).

1.2 SOME ION TRANSPORT ABNORMALITIES IN GENETIC HYPERTENSION

There have been numerous reports of altered intracellular cation concentrations and abnormal handling of ions in both patients with essential hypertension and spontaneously hypertensive rats. These changes in ion handling in genetic hypertension are presented in Table 1.1. Because cell volume and pH regulation requires close control of sodium, potassium and hydrogen ion concentrations in the cell and as changes in the handling of these cations are found in hypertension, it follows that the processes involved in altering ion transport in hypertension have to be linked in some way to both cell volume and pH regulation. This will be discussed later in Chapter 10 of this thesis.

1.3 SOME PROBLEMS WITH *IN VITRO* MEASUREMENT OF ION TRANSPORT

Over the years numerous hypotheses have been suggested to try and explain why changes in ion handling of the cell occurs in genetic hypertension. However, a problem that has faced researchers trying to produce a unifying hypothesis linking ion transport abnormalities and

Table 1.1 Reports of abnormal cation transport and intracellular cation concentrations in genetic hypertension

Variable	Change	Reference
intracellular Na^+ concentration	increased	Edmondson et al., 1975; Ambrosioni et al., 1981; Heagerty et al., 1982; Clegg et. al., 1982; Cole, 1983
	unchanged	Canessa et al., 1980; Garay et al., 1980
intracellular K^+ concentration	unchanged	Boon et al., 1986; Aalkjær et al., 1985
free cytosolic Ca^{++} concentration	unchanged	Nabika et al., 1985; Bukoski, 1990; Pritchard et al., 1989
	increased	Erne et al., 1984; Astaire et al., 1989; Lindner et al., 1987
total cell Ca^{++} content	increased	Postnov and Orlov, 1985; Tobian and Chesley, 1966; Speiker et al., 1988

Table 1.1 (cont)

Na^+/K^+-ATPase activity.	increased	Simon et al., 1987;Nielson et al., 1988; Boon et al., 1986
	decreased	Edmondson et al., 1975; Poston et al., 1981; Heagerty et al., 1982
Na^+/Na^+ (Na^+/Li^+) exchange	increased	Canessa et al., 1980; Canessa et al., 1988
Na^+/H^+ antiporter activity	increased	Livne et al., 1987; Kuriyama and Aviv, 1987, Izzard and Heagerty, 1989; for review see Aalkjær, 1990
Sarcolemmal Ca^{++}-ATPase activity	decreased	for reviews see Postnov and Orlov, 1985 and Dominiczak and Bohr, 1990
Ca^{++} influx via L-type Ca^{++} channels	increased	Rusch and Hermsmeyer, 1988; for reviews see Postnov and Orlov, 1985 and Dominiczak and Bohr, 1990
$Na^+/K^+/Cl^-$-cotransport activity.	variable	for a review see Chipperfield, 1986

hypertension is the inconsistency of the reported changes in ion transport in hypertension. This is particularly so for Na^+/K^+-ATPase activity with both increased and decreased activity of this membrane transport system being reported by different groups (for a review see Swales, 1982) .These inconsistencies have made it very difficult to recognize the significance of any given ion transport abnormality.

It has been suggested that these inconsistencies are due to laboratory variation in *in vitro* conditions (Swales, 1982; Boon, 1986; Nielsen, 1988). This may not be the only explanation for these apparent inconsistencies, as will be discussed in Chapter 10, but there are some problems with *in vitro* methods of measuring ion flux which I will now briefly out-line by giving some examples.

Problems with methods of measuring ion flux *in vitro* can be considered under three main headings:

1. Cell preparation.
2. Method of measurement.
3. Interpretation of results.

1.31 *Cell preparation*

Most studies in hypertension have been performed on blood cells because these cells are easily obtained. However, the way these cells are prepared can alter the results obtained. For example, Poston et al., (1982) have shown that polymorphonuclear leucocytes isolated by the

Ficoll/Triosil method have a lower rate constant for sodium efflux than the same cells prepared by differential sedimentation in dextran.

Differences in temperature of the external media can have an effect on ion transport (Schwartz et al., 1975). Differences in the media used may have an unknown effect on ion transport. This is particularly important for cells grown in culture where the media contains unknown growth factors in the form of non-standardized foetal calf serum.

Further problems may also occur if the reason for the ion transport abnormality is a circulating plasma factor. This may be removed during preparation of the cells.

Furthermore, it has also been shown that cells in culture change there phenotypic identity (Chamley-Campbell and Campbell, 1981; Owens et al., 1986; Aalkjær, 1990) with an alteration of cellular ion transport. For example, Bukoski (1990) has shown that the intracellular calcium concentration in the vascular smooth muscle cells of SHRs on primary culture is normal but increases above normal on subsequent passage

1.32 *Method of measurement*

Some studies have used unphysiological manipulation of cells. An example of such a method is that used by Garay and Meyer (1979) where cells are incubated for 20 hours after inhibiting the sodium pump with the non-specific inhibitor 2,5-p-chloromercuribenzene sulphonate (PCMBS). After such a long incubation with a toxic

compound it is very likely that the cell membrane has been damaged.This could lead to experimentally -induced differences in measuremed ion flux across these membranes.

There have been problems in measuring intracellular sodium concentrations. This stems from the large measurement error that results from attempting to measure a low intracellular sodium concentration in the presence of a very high extracellular sodium concentration. Furthermore, there is recent evidence that much of the sodium that was considered to be intracellular is attached to the outside of the cell membrane. This may have led to false reports of elevated sodium in cells obtained from essential hypertensives (for reviews see Simon, 1989 and Simon, 1990).This inability to measure *true* intracellular sodium concentrations may be responsible for some of the inconsistent findings altered Na^+/K^+-pump activity in hypertension. This is because some methods of measuring Na^+/K^+ pump activity in the past have measured sodium efflux from the cell and have calculated Na^+/K^+-ATPase activity on the basis of an accurate measurement of intracellular sodium concentration and sodium efflux from the cell. A falsely elevated intracellular sodium therefore, would result in an apparent reduction in Na^+/K^+-ATPase activity even in the presence of an increased sodium flux.

Recently there has been considerable interest in measuring Na^+/H^+ antiporter activity in hypertension. Much work has been done using the pH sensitive dye bis-carboxyethylcarboxyfluorescein acetoxymethyl ester (BCECF ester). There is a large error in the measurement of

intracellular pH using this method (Davies et al.,1991). This variability could also result in incorrect interpretation. Futhermore, it has been shown that stimulation of Na^+/H^+ antiporter activity results in an alkaline shift in the cell only if the cell is in a non-bicarbonate buffer. However, this alkaline shift in the cell can be completely removed when bicarbonate is present in the culture media (for a review see Aalkjær, 1990).

1.33 *Interpretation of results*

It is clear from the above discussion, that *in vitro* results can be influenced by the experimental method used and may not represent the changes in ion transport that are present *in vivo*. Thus, abnormailities of ion transport found *in vitro* have to be interpreted on the strength of the method used and should not automatically be interpreted as equivalent to the changes in ion transport that occur *in vivo*.

1.4 THE AIMS OF THIS PROJECT

Problems with *in vitro* methods of studying cation transport have prompted the development over the last few years of methods of studying cation transport *in vivo*. These methods have been based on the principle that certain ions which are not found in high concentrations in the body can be used as markers for endogenous ions, for example rubidium for potassium (Boon et al., 1986) and lithium for sodium (Brearley et al., 1988). These techniques have studied ion trans-

port in blood cells *in vivo* although measurement of intracellular cations concentrations were made *in vitro*.

As an extension of these methods of measuring ion transport *in vivo* it was the main aim of this project to determine whether it would be possible to use nuclear magnetic resonance spectroscopy (NMR) to study ion transport, non-invasively and entirely *in vivo*.

However, at the start of this project NMR was not established as a quantitative tool and previous attempts at quantification had all made assumptions regarding measurement of tissue volume. Thus, these methods could not be applied to the measurement of ion concentrations *in vivo* where a measurement of sample volume is required.

The first aim of this project therefore, was to develop new NMR techniques which would overcome the problem of tissue volume measurement and so measure absolute concentrations of ions *in vivo*.

It was hoped that if these new techniques could be established, it would make it possible to the study rubidium (potassium) kinetics *in vivo*. Such a study would determine the nature of the change in Na^+/K^+-ATPase activity *in vivo* in genetic hypertension ie. whether Na^+/K^+-ATPase activity is *reduced* or *increased* in genetic hypertension.

In addition to the study of Na^+/K^+-ATPase activity, it was hoped that NMR could be used to make non-invasive measurements of intracellular pH (Moon and Richards, 1973), in order to study pH con-

trol and Na^+/H^+ antiporter activity in hypertension *in vivo*. The aim was to determine whether reported *in vitro* abnormalities of pH control and Na^+/H^+ antiporter activity found in association with hypertension are also present *in vivo*.

It was hoped that this work would provide a better understanding of the reasons and mechanisms responsible for abnormalities of ion handling in genetic hypertension.

SUMMARY

In this chapter I have outlined the basic mechanisms by which cells control ion movements in order to regulate both volume and intacellular pH. These mechanisms are relevant to the work that will be presented in the following chapters and I believe relevant to the ion transport abnormalities reported in association with hypertension. I have briefly oulined the common ion transport abnormalities that are found in association with genetic forms of hypertension and have discussed some of the problems with *in vitro* methods of measuring ion transport. I have outlined the main aims of this project.

Chapter two

General Methods

2.1 RADIOCHEMICALS

^{86}Rubidium was obtained from Amersham International plc, Amersham, Bucks

2.2 DRUGS AND REAGENTS

Rubidium chloride, amiloride, 4,4´-di-isothiocyanostilbene-2,2´-disulphonate (DIDS) and Isoprenaline were obtained from Sigma Chemicals, Poole, Dorset. Nifedipine was obtained from Bayer UK Ltd, Newbury, Berks.
All laboratory chemicals were of analytical grade.

2.3 METHOD FOR MEASURING ABSOLUTE CONCENTRATIONS OF RUBIDIUM *IN VIVO* USING ^{87}Rb NMR SPECTROSCOPY

2.31 Animal preparation

Male Wistar-Kyoto rats (150-300 g) were obtained from Olac Ltd., Bicester, UK, and were on a normal rat diet. Each rat then had daily intraperitoneal injections of rubidium chloride solution, in a dose of 2 mmol/kg/day for a period ranging between 3 and 14 days. The animals were then anaesthetized with halothane:N_2O:O_2 and were placed in a specially designed radiofrequency coil (Fig. 2.1) in a 1.9 Tesla superconducting magnet. After collection of the NMR spectra from hindquarter muscle the animals were killed by decapitation and

aliquots of hindquarter muscle were excised. The muscle was divided into four 1 g portions. Three portions of muscle were immediately placed in distilled water to prevent loss of tissue water. One sample was lyophilized. The total muscle water content was calculated from the difference between wet and dry weights. The mean tissue water content of the muscle was 75% of the wet weight. The rubidium content of the muscle was measured as described below.

2.32 NMR Spectroscopy

All experiments were performed in a horizontal, wide-bore, 1.9 Tesla magnet at a frequency of 80.28 MHz for protons and 26.28 MHz for rubidium. Signals were obtained from rat hindquarter muscle.

2.321 *The radiofrequency coil*

The earphone coil construction and circuitry are illustrated in Fig. 2.1. Each coil head had an external diameter of 17 mm and the heads were positioned 11 mm apart. Having two surface coils opposed to each other in this way creates a uniform B_1 field across the sample.

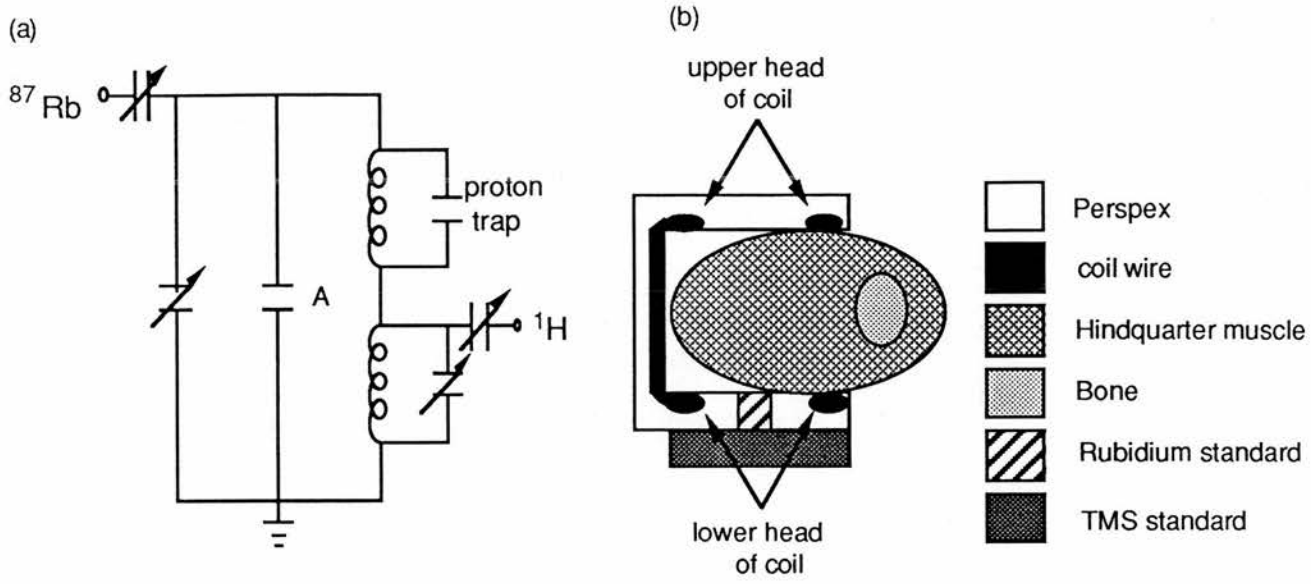


Fig. 2.1 (a) Diagram of the radiofrequency coil circuit. For the purpose of this study the coil was tuned to both ^{1}H and ^{87}Rb, but it can be tuned to other nuclei , including ^{31}P, ^{23}Na and ^{7}Li. This can be done by connecting different capacitors at position A. The same coil could be used to measure all of these nuclei in the same animal. (b) Diagram of the coil in coronal section, showing the relationship between the sample tissue and standards.

When the B_1 field is uniform the signal received by the coil from the sample lying in a uniform part of the B_1 field, immediately after a 90° pulse, is proportional to b can be expressed by the following equation:

$$\text{e.m.f.} = \omega_0 B_1 M_0 V \qquad (1)$$

where e.m.f. is the electromotive force created in the coil by the sample magnetization, ω_0 is the angular velocity of the nucleus concerned, B_1 is the the stimulating magnetic field created by unit current passing through the radiofrequency coil, M_0 is the bulk component of magnetization per unit of sample volume and is proportional to the number of resonant nuclei in the sample, and V is the volume of sample.

2.322 *Spectral parameters*

For the purpose of this experiment the coil was tuned to both rubidium and protons, but it could be tuned to other ions, including sodium and lithium. NMR spectra were collected as 512 data points with a sweep width of 10 000 Hz for rubidium and as 1024 data points with a sweep width of ±600 Hz for protons. Pulse widths were obtained at 90^{o} for rubidium, water, and tetramethylsilane (TMS). The mean pulse width for rubidium was 15 μs and for protons 22 μs. The water standard, TMS, experienced a smallerB_1 field, due to its position relative to the centre of the coil, and as a result had a much longer pulse width of 55 μs. In muscle tissue the T_1 relaxation time for rubidium was found to be 500 μs and the T_2 relaxation time 350 μs.

2.33 Predicted NMR visibility

The theoretical predicted NMR visibility of rubidium in muscle using a 1.9 Tesla magnet can be expressed by the following equation (Torrey 1949):

$$M_{vis}/M_{tot} = 0.4 \{\exp(-D_E/T_{2*}) \exp [-(1/T_1 + 1/T_{2*})t_p]\} \quad (2)$$

where M_{vis} is the detectable magnetization from rubidium nuclei in muscle tissue; M_{tot} is the total magnetization from rubidium nuclei in muscle tissue; D_E is the dead time of the spectrometer in μs (50 μs); t_p is the pulse width in μs (14-15 μs)

The factor of 0.4 in this equation arises from the fact that the nucleus of ^{87}rubidium is quadrupolar, of spin -3/2. Compared with other spin -3/2 nuclei, its NMR relaxation times are short (in aqueous solution $T_1 = T_2 = 2.5$ ms (Allis et al 1989). However, in anisotropic biological environments it exhibits complex relaxation behaviour. Interactions with both free and membrane-bound proteins and other macro-molecules cause shortening of the relaxation times. As the line width is inversely related to the transverse relaxation time (T_2), the lines are correspondingly broad. Furthermore, in anisotropic environments spin -3/2 nuclei are characterized by two T_2 times: 60 % of the magnetization relaxes rapidly (T_2 fast) and 40 % relaxes more slowly (T_2 slow). These relaxation times depend both on the environment of the nuclei and the magnetic field strength. These relaxation effects are not specific to rubidium and are seen in other spin -3/2 nuclei, such as ^{23}Na

and ^{39}K (Forsen and Lindman, 1981; Deverell and Richards, 1966). In a 1.9 Tesla magnet the fast component of transverse relaxation is too broad to be detected in the spectra. The remaining 40 % of the magnetization has a T_2 of about 350 μs, and this gives the 900-1000 Hz line width of the above muscle spectrum.

A further reduction in signal intensity arises from relaxation during the radiofrequency pulse and the spectrometer dead-time, according to equation 2 (Torrey, 1949). This reduces the expected visibility from 40% to 33%, taking into account the 3% attenuation of the signal from the calibration solutions (see next section). Thus, although magnetization is detected from all the rubidium nuclei in the *anisotropic* environment of muscle, the spectrometer detects only 33% of the magnetization that would be detected in free solution (an *isotropic* environment) from the same number of nuclei.

2.34 NMR standards

2.341 *Rubidium*

The rubidium standard consisted of 45 μl of a 0.5 M solution of RbI in saturated KI solution, sealed in a glass vial. Iodide was used as a shift reagent, since of the alkali halides it gives the best shift for rubidium (Fosen and Lindman, 1981). The shift achieved was 55 ppm to a higher frequency than rubidium without iodide (see Chapter 3, Fig. 3.1a).

2.342 *Water*

The water standard consisted of 2 ml of tetramethylsilane (TMS) in a sealed glass vial, giving a proton shift of 5 ppm to a lower frequency than water(see Chapter 3; Figs. 3.2b and c).

2.343 *Calibration*

Both the rubidium and water standards were calibrated with phantoms of rubidium solutions of different volumes up to 50 ml (ie several times larger than the volume of a rat's hindquarter). The rubidium peak area in muscle was compared with the rubidium standard peak area, and the number of μmol of rubidium in the muscle was obtained from a calibration graph.

Tissue water was calculated in a similar manner from the peak areas of the proton spectra of both tissue water and TMS. All areas were measured by cutting and weighing plotted spectra.

2.35 Atomic Absorption Spectrophotometry

The 1 g portions of muscle were homogenized in deionized water using an Ultraturrax (Model TP18 - 10). The muscle suspension was spun in a Mistral MSE 4L centrifuge (1400 **g** at 4^{o}C for 15 min) and the supernatant was collected. The muscle pellet was resuspended and this procedure was repeated twice, the supernatant being collected each time. The pooled supernatant was then spun in a Sorvall RC-5B

ultracentrifuge (Du Pont instruments) at 40 000 **g** for 15 min. The resulting clear supernatant contained 90% of the total tissue rubidium as assessed from recovery experiments using radioactive ^{86}Rb. This final supernatant was diluted to an appropriate level to enable measurement of the final rubidium concentration in triplicate by flame atomic absorption spectrophotometry (Varian Atomic Absorption Spectrophotometer, Model SpectrAA 300/400). The rubidium concentration of the muscle was expressed as mmol/l of tissue water

Whole rat blood (5-10 ml) from each animal was spun in a Mistral MSE 4L centrifuge (1400 **g** at 4°C for 15 min) to separate the erythrocytes and plasma. Appropriate dilutions were made to enable measurement of the rubidium concentrations in triplicate by flame atomic absorption spectrophotometry (Varian Atomic Absorption Spectrophotometer, Model SpectrAA 300/400). Rubidium concentrations were expressed as mmol/l of plasma and mmol/l of packed erythrocytes.

2.4 METHOD USED TO MEASURE Na^+/H^+ ANTIPORTER ACTIVITY *IN VIVO*

2.41 Animal preparation

Male 13-week old SHRs and age-matched WKY in-bred controls, ranging in weight from 240 g to 312 g, were obtained from Olac Ltd, Bicester, UK. Anaesthesia was induced with pentobarbital sodium (30 mg/kg) and was maintained during each experiment with halothane

(0.5-1.0 % in 1:1 N_2O:O_2) delivered through a face-mask. Sciatic nerve stimulation was used to cause contraction of the plantaris, gastrocnemius, and soleus muscles as a group. The method of preparing the rats for stimulation of the plantaris-gastrocnemius-soleus muscle complex has been described previously (Schoubridge et al 1984). Glycolytic fibres (types IIa and IIb) are the main constituent of these muscles (Armstrong and Phelps 1984). Briefly, the left sciatic nerve was exposed and a platinum electrode was placed in contact with the nerve. Brass pins were driven through the knee joint and ankle joint and the leg was immobilized in a specially-designed Perspex cradle. The distal tendon of the plantaris-gastrocnemius-soleus complex was attached to an isometric force transducer and the output was displayed on a polygraph. The length of muscle which gave maximum isometric twitch tension in response to a supramaximal sciatic nerve stimulation was then determined.

The stimulation protocol consisted of pulses of 50 μs pulse-width at 30 V, at a frequency of 10 Hz. The muscle was stimulated for 20 min. Mean arterial pressure was monitored by an indwelling catheter in the external carotid artery and was 120 (SD 9.4) mmHg in the WKY rats (n = 20) and 185 (SD 8.9) mmHg in the SHRs (n = 20).

2.42 NMR spectroscopy

All experiments were performed at a Larmor frequency of 73.8 MHz for ^{31}P, in a vertical wide-bore 4.3 Tesla magnet. The radiofrequency coil, which acted as both a transmitter and a receiver, was a Helmholtz

coil, each head having an external diameter of 15mm. The coil surrounded the plantaris-gastrocnemius-soleus group of muscles of the rat leg. Spectra were collected as 2K data points using a sweep width of ± 2000 Hz. Two resting spectra were obtained, one using a nominal tip angle of 90° and another at a tip angle of 60°. Spectra obtained during muscle contraction were all obtained using a tip angle of 60°. Spectra were quantified by computer line-fitting, and the pH was determined from the chemical shift of P_i from PCr, which was nominally given a shift of 0 p.p.m. (Moon and Richards, 1973). The acquisition trigger of the spectrometer was gated such that 32 spectra were collected every 80 s. The acid measurements thus obtained represented the average muscle pH during sequential 80 s intervals of muscle contraction.

2.43 Measurements of metabolite concentrations

Blood and muscle samples were taken from animals both at rest and after muscle stimulation for 2 min. Blood samples (0.5 ml) were taken from the indwelling external carotid artery cannula. Muscle samples were rapidly excised, immediately freeze-clamped in pre-cooled aluminium tongs, and stored in liquid nitrogen. Muscle and blood lactate concentrations were measured using standard biochemical techniques (Bergmeyer, 1974). Muscle glycogen was measured after digestion of the muscle in 1 M KOH and neutralization of the solution in glacial acetic acid; glycogen was converted to glucose by incubation with amyloglucosidase (in acetate buffer, pH 4.8) for 2 h at 37 °C and the glucose was measured enzymatically (Bergmeyer, 1974). Resting ATP,

phosphocreatine (PCr), and free creatinine concentrations were measured using hplc. All metabolite concentrations are reported on a dry weight basis. The mean wet/dry weight ratio was 4.4 (SD 0.22) for both SHRs and WKY rats at rest and after stimulation for 2 min. There was no significant difference in the wet/dry ratio after any treatment. Absolute concentrations of phosphorus metabolites in the ^{31}P NMR spectra were calculated assuming complete visibility of ATP and PCr (Dawson and Wilkie, 1980). The concentration of ATP at rest was taken to be that of the concentration found using hplc and PCr calculated from resting fully relaxed spectra. The concentrations of the other metabolites at rest were calculated from the resting spectra, using the ratio of the spectral peak of the metabolite to that of PCr. Absolute concentrations of metabolites during contraction were then calculated by comparing the absolute intensity of the respective signal of each metabolite in a given spectrum during contraction to that of the resting spectrum, taking into account the saturation effect resulting from a 60° flip angle.

2.5 Statistical and mathematical methods

Tests of normality were performed on all data (Royston, 1983). Results were analyzed using unpaired Student's t-tests and one-way ANOVA applying Dunnett's test and the Fisher's Protected Least Significant Difference test where appropriate. Areas under curves (AUCs) were calculated using Simpson's method.

Chapter Three

Measurement of concentrations of rubidium *in vivo* in rat skeletal muscle by ^{87}rubidium NMR spectroscopy

3.1 INTRODUCTION

Rubidium, one of the Group 1 alkali metals, has been shown to substitute for potassium in a variety of transmembrane transport systems, and has been used to study the activity of the sodium- and potassium-activated adenosine triphosphatase (Na^+/K^+-ATPase, E.C. 3.6.1.37) (Bernstein and Israel, 1970; Love and Burch, 1953) and of Na^+/K^+ co-transport (Chipperfield, 1986). Like potassium, rubidium is concentrated in the intracellular compartment, the ratio between the intracellular and extracellular compartments being about 30:1 (Relman, 1956; Meltzer and Fieve, 1975; Boon et al., 1984). *In vitro* muscle Na^+/K^+-ATPase activity has been measured using rubidium kinetics in several conditions, including thyroid disease, obesity, old age, and hypertension (Clausen,1986). In hypertension in particular, *in vitro* studies of Na^+/K^+-ATPase activity and Na^+/K^+ co-transport have resulted in conflicting results and diverse opinions as to the importance of these ion transport abnormalities (Swales, 1982). Differing methods of measuring Na^+/K^+-ATPase activity *in vitro* may be the cause of the confusing results in hypertension.

The particular example of hypertension emphasises the need for non-invasive methods of measuring *in vivo* Na^+/K^+-ATPase activity. This has previously been done in man by studying the disposition of rubidium in plasma and erythrocytes after an oral load of rubidium chloride (Boon et al., 1984). In order to extend this work to other tissues a method has now been developed to make *in vivo* measurement of ru-

bidium concentrations in skeletal muscle using nuclear magnetic resonance spectroscopy (NMR).

In order to make absolute measurements of a substance by NMR many problems need to be overcome. These problems are compounded *in vivo* (Tofts and Wray, 1988). To date most *in vivo* NMR studies have been directed towards the measurement of *relative* concentrations of phosphorus metabolites (Radda, 1986), using radiofrequency surface coils that can be placed on the surface of an animal (Ackerman et al., 1980). Although the development of the surface coil has made *in vivo* NMR much easier, the use of surface coils leads to problems in quantification, the main problem being that a surface coil does not have a uniform magnetic (B_1) field. Different parts of the sample therefore give rise to signals of different intensity, making it very difficult to obtain absolute concentrations. This problem can be overcome if a suitable internal standard of the nucleus of interest is present in the cell. Provided the concentration of this standard is known accurately, is not involved in cell compartmentation, and does not change with time, the ratio of this standard to the compound of interest can give an estimate of tissue concentration. One such standard is naturally-occurring deuterium oxide (^2H_2O) (McCoy et al., 1988). However, for most of the commonly studied NMR nuclei no such standard exists. Several methods have been used to try to overcome the problem of a non-uniform B_1 field, but with all of these methods some form of assumption has been made regarding sample volume (Thurlborn and Ackerman, 1983; Roth et al., 1989; Wray and Tofts, 1986).

A further complication of *in vivo* NMR measurement concerns electrical conduction losses in living tissues (Wray and Tofts, 1986). These losses attenuate the signal received by the NMR spectrometer in a sample-dependent manner. Conduction losses are greater in larger animals and could lead to differences in the signal obtained, independent of the number of nuclei present in the sample.

^{87}Rubidium, a non-radioactive isotope of rubidium, which comprises 30% of natural abundance rubidium, has recently been the subject of both *in vitro* and *ex vivo* NMR studies (Helpern et al., 1987; Allis et al., 1989). The aim of this work was to develop a method of measuring absolute concentrations of both tissue rubidium and water, non-invasively in intact animals, using NMR spectroscopy in a way that would overcome the problems outlined above. By using this non-invasive method of measuring tissue rubidium concentration, it would then be possible to measure dynamic changes in tissue rubidium in the same animal, and thus to assess Na^+/K^+-ATPase activity *in vivo*. Measuring changes in tissue rubidium concentration allows assessment of Na^+/K^+-ATPase transport activity, since well over 90% of tissue rubidium is intracellular and 90% of cellular rubidium uptake occurs as a result of Na^+/K^+-ATPase activity (Bernstein and Israel, 1970; Overbeck et al., 1981).

The principle approach has been to use a coil tuned to both ^{87}Rb and ^{1}H [20], and to measure the total amount of rubidium seen by the coil and the total amount of water seen by the coil in the same volume of

tissue. The ratio of the two then gives a measure of *absolute* tissue rubidium concentration.

3.2 METHODS

(see Chapter 2)

3.3 RESULTS

3.31 Spectra

Fig. 3.1a shows a typical muscle spectrum, with a narrow peak arising from the rubidium standard, shifted 55 p.p.m. to the left, overlapping a broad peak obtained from muscle rubidium. These peaks are sufficiently shifted from each other to enable measurement of the areas of both peaks. Fig. 3.1b shows that the peaks resulting from the protons of tissue water, tissue fat, and the proton standard Tetramethylsilane (TMS) are shifted from each other such that overlap does not occur. Fig. 3.1c shows that when a 90^0 pulse is applied to the TMS, the water peak is inverted. There is a small fat peak between the peaks of water and TMS, and this does not contribute to the area of either of these peaks.

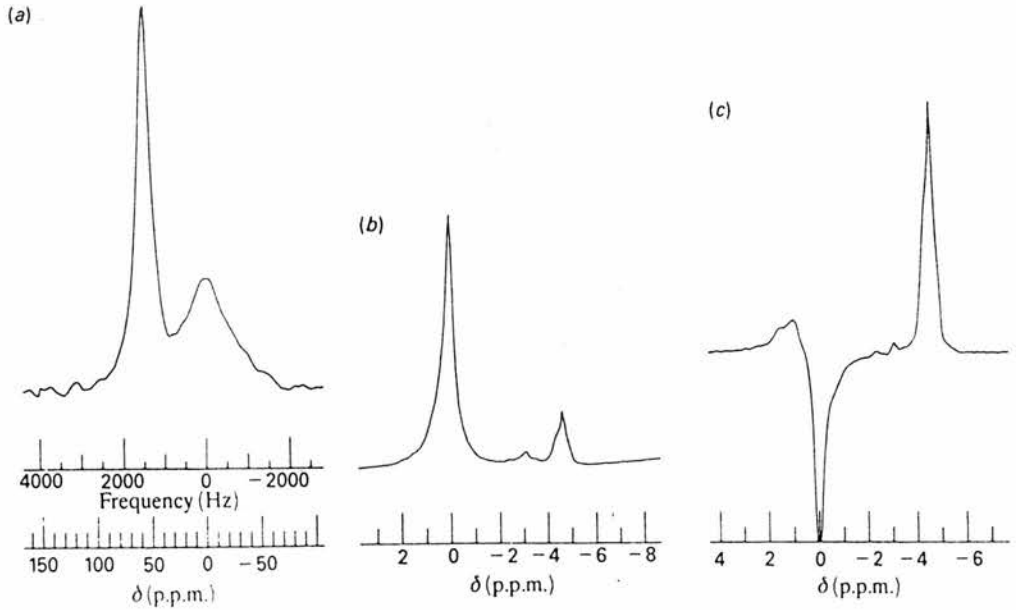


Fig. 3.1 (a) Rubidium spectrum obtained *in vivo* from hindquarter muscle of a rubidium-loaded rat. The broad peak of 1000 Hz is the signal received from tissue rubidium. The narrow peak which is shifted 55 p.p.m. to the left of the muscle peak is the signal obtained from the rubidium standard. (b) Proton spectrum obtained at the 90^0 pulse width for tissue water. The large peak shown (0 p.p.m.) is tissue water, the peak at -5 p.p.m. is TMS, and a further small peak at -3 p.p.m. is tissue fat. (c) Proton spectrum obtained at the 90^0 pulse width for TMS. The inverted peak shown is tissue water (0 p.p.m.). The TMS peak is shifted to the right (-5 p.p.m.). A peak obtained from tissue fat (-3 p.p.m.) can be seen between the peaks of tissue water and TMS.

3.32 The effect of increasing conductivity

In a series of experiments latex phantoms were filled with solutions of rubidium chloride and sodium chloride. The rubidium concentration was constant at 20 mmol/l and sodium chloride was added to give sodium concentrations of 150 mmol/l to 4.8 mol/l. This gave solution conductivities ranging from 1.4 to 192 mS. From Fig. 3.2 it can be seen that increasing conductivity had no effect on rubidium concentration estimation. This result was obtained despite a reduction in the peak area from both the sample and standard. Increasing conductivity reduces the efficiency of the coil as both a transmitter and a receiver. As both the sample and the standard are in a fixed physical relationship relative both to each other and to the coil, reduced coil efficiency attenuates the signals from both the sample and the standard to a similar degree, and the peak area ratio between the two remains constant. If such ratios are used in the measurement, conductivity, and for similar reasons variations in tuning and amplifier gain, can have no effect on rubidium concentration estimations.

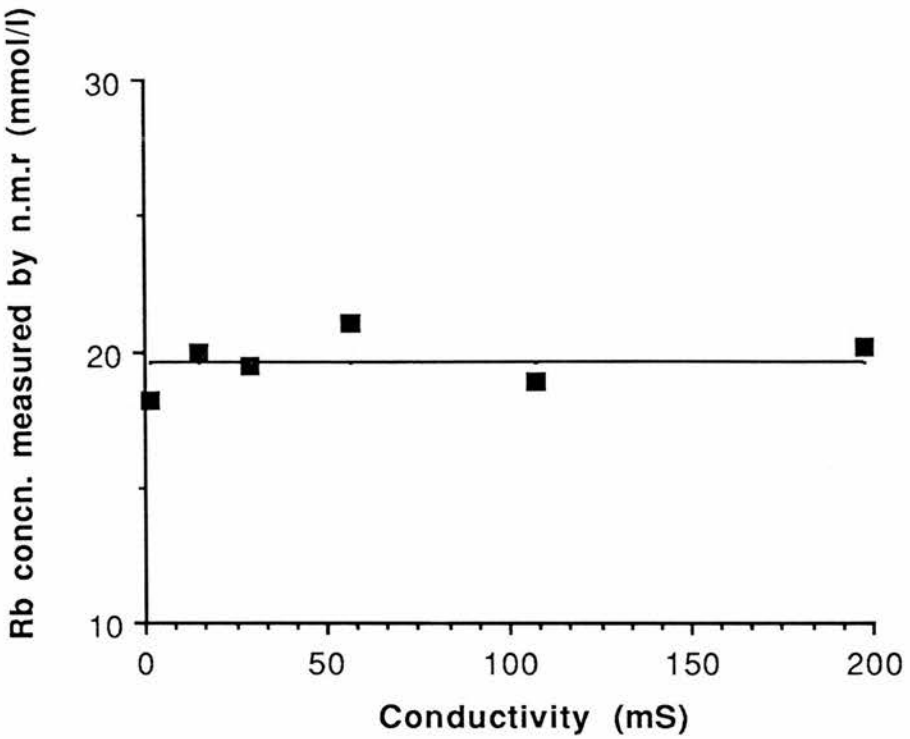


Fig. 3.2. Effect of increasing conductivity on the measurement of rubidium concentration using latex phantoms filled with the same volume of 20 mmol/l rubidium chloride solution.

3.33 Measurement of volume by NMR

Latex phantoms of various volumes, filled with 30 mmol/l rubidium chloride solution, were placed in the coil, and NMR spectra were obtained for both rubidium and water. Fig. 3.3a shows that the water/TMS proton ratio increased linearly until the sensitive volume of the coil (2.7 ml) was exceeded. Above this volume no further increase in the ratios resulted.

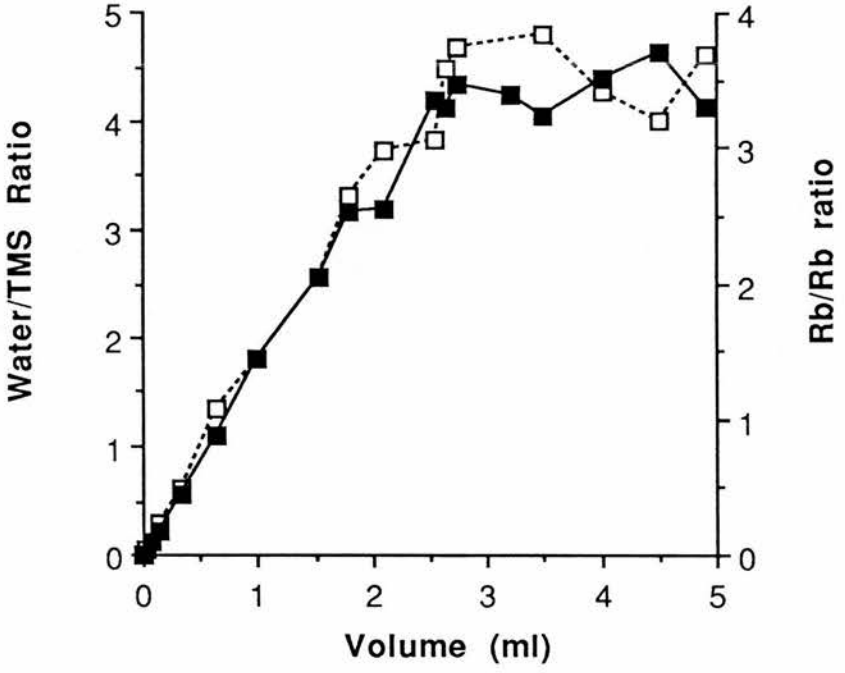
Fig. 3.3a also shows that the rubidium/rubidium ratio increased linearly until the sensitive volume of the coil (2.7 ml) was exceeded. The exact overlap of the rubidium/rubidium and the water/TMS proton ratios shows that the rubidium detected by the coil is contained only within water which the coil also detects.

Fig. 3.3b and Fig. 3.3c show the respective calibration curves for water and rubidium derived from this volume experiment.

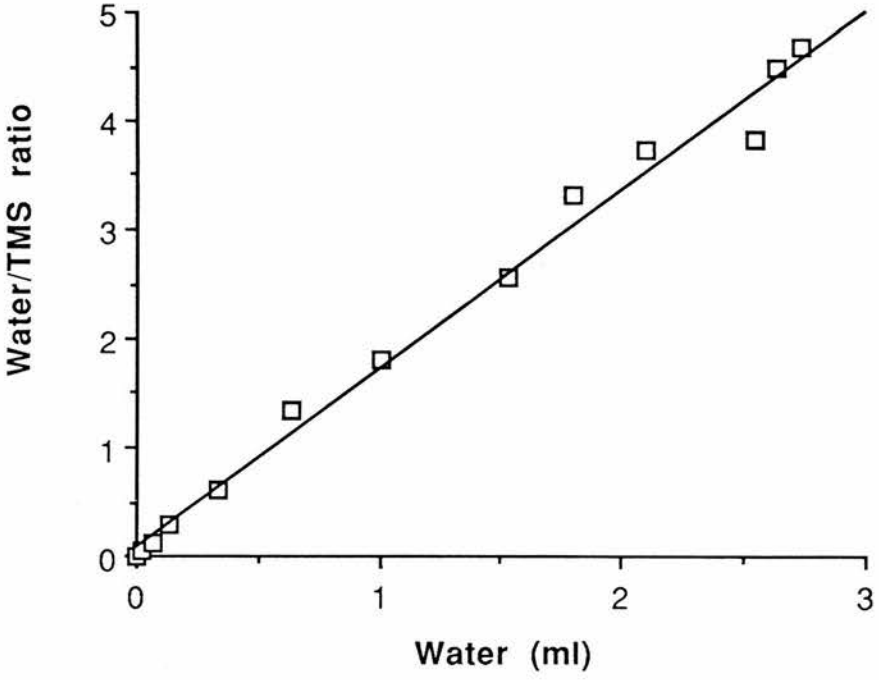
3.34 NMR visibility of rubidium in living tissue

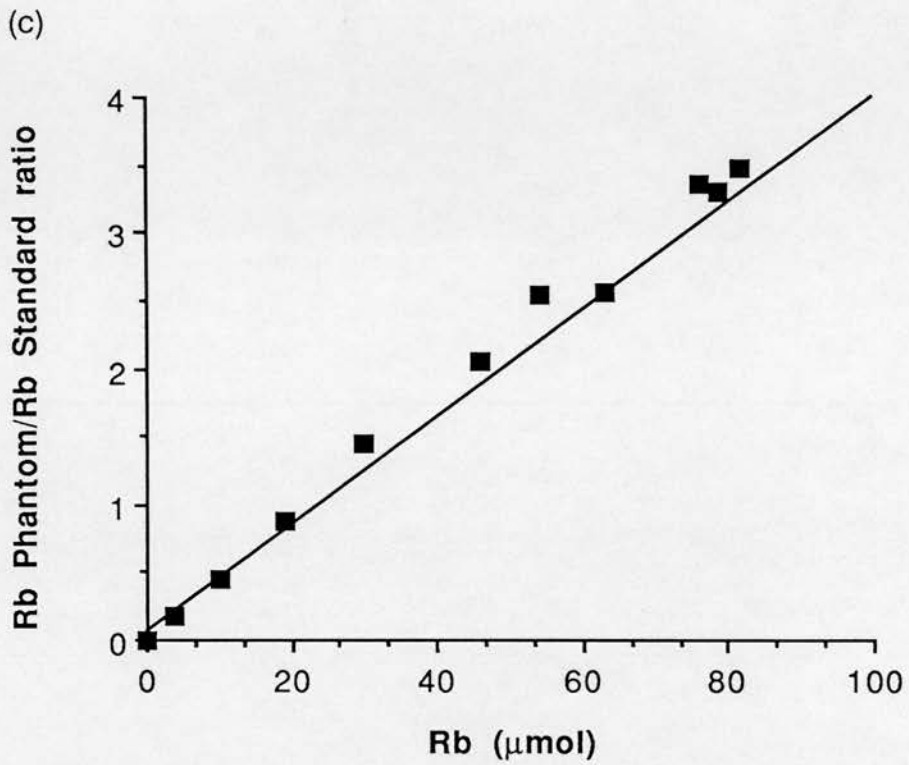
Table 3.1 shows the NMR estimates of tissue rubidium content and water volume calculated from spectra obtained from the hindquarter muscle of live rats under anaesthesia. It also shows the mean rubidium concentration of excised muscle obtained from the same animals, as estimated by flame atomic absorption spectrophotometry. It can be seen from these results that the volume of tissue water in these animals is on the linear part of the volume calibration curve. The tissue rubidium concentration for each animal as determined by NMR was found to be 0.31 (SEM 0.04) of that of the rubidium concentration estimated by flame atomic absorption spectrophotometry. This is close to the predicted NMR visibility of 0.33 obtained from equation 2.

(a)


Water/TMS Ratio
Rb/Rb ratio
Volume (ml)
0
1
2
3
4
5

(b)


Water/TMS ratio
Water (ml)
0
1
2
3
4
5



Figs. 3.3

(a) Effect of filling the coil with increasing volumes of a 30 mmol/l rubidium solution on the water/TMS proton ratio (□----□) and on the sample rubidium/rubidium standard ratio (■——■). (b) Calibration curve for increasing amounts of sample water placed in the coil, plotted against the water/TMS proton ratio. y=0.08 + 1.66x; r=0.990. (c) Calibration curve for increasing amounts of sample rubidium placed in the coil, plotted against the rubidium sample/rubidium standard ratio. y=0.05 + 0.042x; r=0.994.

Table 3.1 Estimation of tissue rubidium by NMR and flame atomic absorption spectrophotometry, together with measured NMR visibility

Abbreviation: FAAS, flame atomic absorption spectrophotometry. NMR, nuclear magnetic resonance spectroscopy.

Rat no.	Weight (g)	N MR Rb (μmol)	Tissue water (ml	Tissue Rb concn. by NMR (mmol/l)	Tissue Rb concn. by FAAS (mmol/l)	NMR visibility (%)
1	268	16.7	1.9	8.8	26.7	33
2	287	9.3	1.8	5.2	16.9	31
3	303	7.0	1.9	3.7	15.5	24
4	267	7.9	1.6	4.9	15.5	32
5	288	7.4	2.0	3.7	14.7	25
6	288	5.8	1.8	3.2	10.6	30
7	355	22.1	2.0	11.1	34.0	33
8	350	21.4	2.2	9.7	31.5	31
9	360	26.2	2.2	11.9	29.4	40
10	355	22.6	2.2	10.3	29.7	35
11	345	15.5	2.3	6.7	24.0	28
12	342	18.3	2.2	8.3	27.2	31

3.4 DISCUSSION

It has been shown here that it is possible to measure rubidium concentrations in rat skeletal muscle *in vivo*, by measuring the muscle content of both rubidium and water in the same sample of tissue.

One problem with using NMR to make biological measurements is its relative insensitivity. Although rubidium is found in trace amounts in biological tissues, in order to achieve detectable concentrations of rubidium in tissues using NMR it has to be given exogenously. This has been done using other NMR visible nuclei, including ^{7}lithium, ^{13}carbon, and ^{19}fluorine (Avison et al., 1986; Burt et al., 1986). In this study the lower limit of detection of tissue rubidium concentration was 10 mmol/l. Even with larger coils, increasing the sensitivity of detection of rubidium, it is unlikely that satisfactory spectra will be achieved with tissue concentrations of less than 1mmol/l. This may still not be a problem for the future study of tissue rubidium disposition *in vivo* in man and in animals, since rubidium is well tolerated in rat and man. In this study tissue concentrations of over 30 mmol/l were achieved without any adverse effects. In man large doses of rubidium have been given orally, also without adverse effects (Meltzer and Fieve, 1975; Fieve et al., 1973). The concentrations reached in the plasma in such studies in man would result in tissue concentrations well within the bounds of detectability with NMR, in view of the known intracellular:extracellular rubidium concentration ratio (Meltzer and Fieve, 1975).

The coil design (Fig. 1) ensures a uniform B_1 field across the sample for the *in vivo* quantification of skeletal muscle rubidium concentrations by NMR, and this is an essential feature of the method. In contrast, it is not necessary for the standard also to be in a uniform B_1 field. A non-uniform B_1 field can result in consistent spectral areas, provided that a 90^0 pulse width is used to stimulate the sample and that differences in conductivity, amplifier gain, and tuning are negligible from day to day (Tofts and Wray, 1988). However, significant day-to-day differences *do* occur, but this problem has been overcome by maintaining a constant physical relationship between the sample, standard, and coil, and by using ratios of signals from the sample and the standard. The TMS, used as a standard for tissue water, was situated under the bottom head of the coil, and was therefore in a non-uniform B_1 field. However, the essential point to note about this method is that the absolute intensity of the signals from both the standard and the sample can change with alterations in conductivity, amplifier gain, and tuning, without altering the ability of the method to measure absolute concentrations. This is so because when the sample and standard are in a constant physical relationship both to each other and to the coil alterations in coil sensitivity will change the signals obtained from both the sample and the standard proportionately, and thus the ratio of the spectral areas will remain constant. This has been clearly shown in this study in relation to conductivity (Fig. 3.2). Thus, all that is required of a standard using this method is that it should have a constant physical relationship to both the coil and the sample, and that its spectral peak should be of a suitable size compared to the sample peak.

Using this method it should now be possible to study the rates of movement of rubidium into and out of skeletal muscle *in vivo* , thus enabling direct measurements of the *in vivo* activity of the transport systems responsible for these movements, such as the Na^+/K^+-ATPase

3.5 CONCLUSIONS

In this study nuclear magnetic resonance (NMR) spectroscopy has been used to measure rubidium concentrations in the skeletal muscle of live intact rats. Using a 1.9 Tesla superconducting magnet and an ear-phone coil tuned to both protons (^1H) and rubidium (^{87}Rb), it was possible to make measurements of both tissue rubidium content and water content, and from these measurements to obtain rubidium concentration.

The NMR estimate of rubidium concentration in muscle *in vivo* was found to be a constant 31 % (SEM 4 %) of that estimated by flame atomic absorption spectroscopy in an extract of excised muscle. This is close to the predicted theoretical NMR visibility of 33 %. The visibility was constant for muscle rubidium concentrations ranging between 10 and 34 mmol/l.

Rubidium concentration measurement by this method is unaffected by variations in sample geometry, sample volume, tissue conductivity, coil tuning and amplifier gain.

By using this method to measure changes in tissue rubidium concentration with time in the same animal, it should now be possible to assess the *in vivo* activity of ion transport systems, such as sodium- and potassium-activated adenosine triphosphatase (Na^+/K^+-ATPase), by measuring the rates of change of tissue rubidium concentrations during the administration of rubidium salts.

This method could also be used to measure the absolute concentration of any NMR visible nucleus and could be applied to man.

Chapter four

Na^+/K^+-ATPase activity *in vivo* in the SHR

4.1 INTRODUCTION

It has been postulated that in essential hypertension inhibition of Na^+/K^+-ATPase activity in vascular smooth muscle cells could result in increased vascular tone (Blaustein, 1977), although a convincing mechanism for such an effect is lacking (Swales, 1982). Two types of evidence have been adduced in support of this hypothesis. Firstly, the presence in the plasma of increased concentrations of an inhibitor of the Na^+/K^+-ATPase (for a review see De Wardener and MacGregor, 1985). Secondly, reports of decreased Na^+/K^+-ATPase activity, for example in the leucocytes of patients with essential hypertension (Edmondson et al., 1975).

However, an endogenous inhibitor has also been reported in association with other diseases, such as chronic renal failure, in which the blood pressure is not necessarily raised (Cole et al., 1968). Furthermore, several workers have demonstrated *increased* rather than *decreased* ion transport activity attributable to the Na^+/K^+-ATPase in erythrocytes from patients with essential hypertension (Simon and Engel, 1987), in the leucocytes of their first-degree relatives (Nielson et al 1988), and in vascular smooth muscle in SHRs (Pamnani et al., 1980).

In addition to the problems raised by the inconsistency of these reports, there is the major problem that *in-vitro* studies may not reflect the true nature of any change in Na^+/K^+-ATPase activity which may have occurred *in vivo*. This has led to the development of methods of studying *in vivo* Na^+/K^+-ATPase activity in hypertension. In man this

has previously been done by studying the disposition of potassium and the potassium substitute rubidium after intravenous and oral administration with the finding that *in-vivo* potassium and rubidium influxes are increased in the erythrocytes of patients with essential hypertension (Boon et al., 1986). Extending these *in vivo* studies to a tissue other than erythrocytes a novel non-invasive method of measuring absolute intracellular non-radioactive rubidium concentrations in intact animals by ^{87}Rb nuclear magnetic resonance spectroscopy (^{87}Rb-NMR) has been developed (see Chapters 2 and 3).

Rubidium has been used for many years as a potassium substitute in studying potassium fluxes (Bernstein and Israel, 1970; Love and Burch, 1953) and over 90% of cellular rubidium influx occurs via Na^+/K^+-ATPase activity (Bernstein and Israel, 1970; Overbeck et al., 1981). Thus, the *in-vivo* rate of accumulation of rubidium in cells can be used as an index of Na^+/K^+-ATPase activity. Sequential measurements of skeletal muscle rubidium concentrations were made, during the administration of rubidium chloride (RbCl) to SHRs and WKY rat controls, in order to measure the rate of rubidium uptake into skeletal muscle in hypertensive and normotensive animals and so to assess Na^+/K^+-ATPase activity in these animals.

Skeletal muscle was chosen because it was not possible to study vascular smooth muscle *in vivo* using ^{87}Rb-NMR. SHRs were chosen because they have been shown to be a good model of essential hypertension (Frolich, 1986), and abnormalities in ion transport, at least *in vitro*, are reportedly similar in SHRs and in patients with essential hypertension (Orlov et al., 1989).

4.2 METHODS

4.21 Animals

15 male 13-week old SHRs, mean (SD) blood pressure (BP) 180 (10) mmHg, and 15 male age-matched WKY inbred controls, mean BP 120 (10) mmHg, were obtained from Olac Ltd, Bicester, UK. Animals were maintained on a normal diet. There was no significant difference in mean body weight between the SHRs and the WKY rats at any time during the study. Each animal was given a daily dose of 2 mmol/kg of RbCl intraperitoneally. It has previously been shown that rubidium is rapidly and completely absorbed in both SHRs and WKY rats within 90 minutes of an intraperitoneal injection (Boon et al 1983). Repeated ^{87}Rb-NMR measurements of muscle rubidium concentrations were made daily over a period of 3 weeks, each rat contributing three measurements in all. Rubidium administration was then continued for a further week, by which time steady-state tissue concentrations had been reached. RbCl injections were then stopped and further ^{87}Rb-NMR measurements of muscle rubidium concentrations were made at various intervals, in different animals, up to one week after the last injection. At different times after withdrawal, animals were killed and plasma and intra-erythrocytic rubidium concentrations were measured by flame atomic absorption spectroscopy. Halothane:N_2O:O_2 anaesthesia was maintained during ^{87}Rb-NMR measurements of muscle rubidium concentrations. Five rats in each group died towards the end of the loading period and the data they contributed have been included.

4.22 NMR Spectroscopy

Muscle rubidium concentration measurements were made by measuring the absolute amounts of both rubidium and water in the muscle as previously described (see Chapters 2 and 3).

4.23 Atomic Absorption Spectrophotometry

Erythrocytic and plasma rubidium concentrations were measured as previously described (see Chapter 2).

4.3 RESULTS

Fig. 4.1 shows the increases in muscle rubidium concentrations in both the SHRs and the WKY rat controls, during daily injections of 2 mmol/kg rubidium chloride (RbCl). There was a higher rate of rise of rubidium concentrations in the SHR skeletal muscle, but the same steady-state concentration of 45 mmol/l was achieved in both SHRs and WKY rats (95% CI 33-54 for SHRs and 40-54 for WKY rats). Using the values of λ_{muscle} calculated from the rates of disappearance of rubidium from skeletal muscle, as shown in Fig.4.2b and as discussed in detail below, the theoretical curves of appearance of rubidium in skeletal muscle which should have occurred during the loading phase were calculated. These curves are shown in Fig. 4.1 as continuous lines.

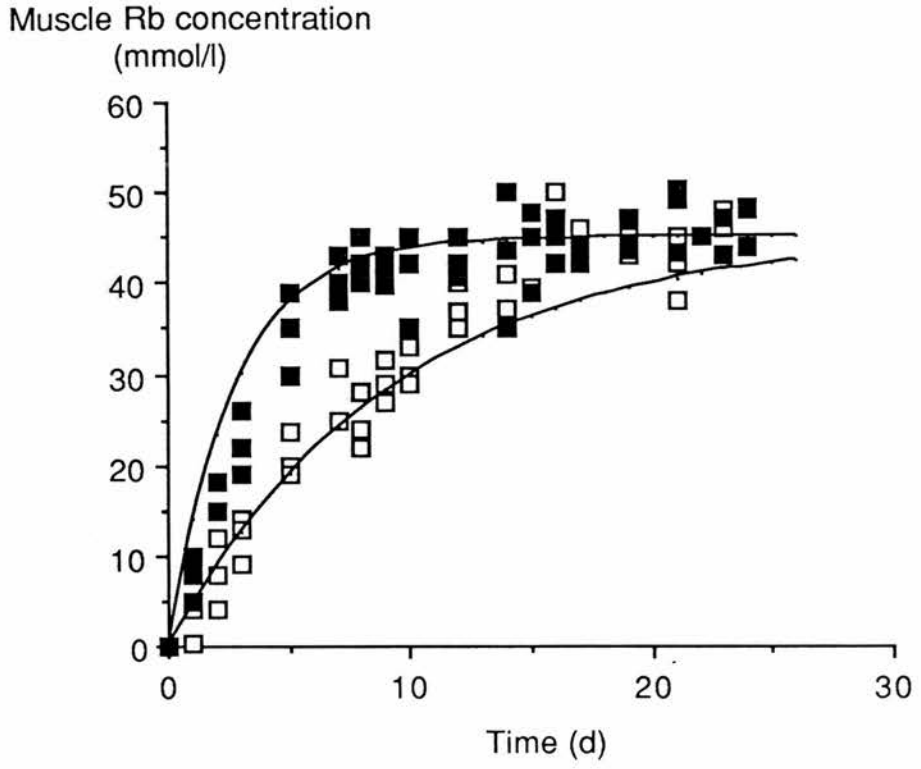


Fig. 4.1 Skeletal muscle rubidium concentrations measured by ^{87}Rb-NMR. in spontaneously hypertensive rats (■) and Wistar-Kyoto rats (□) during repeated daily administration of RbCl. The lines which have been drawn through the data points have been derived from equation 1 in the text, using values of the elimination rate constant of rubidium in the muscle derived from the data shown in Fig. 4.2.

Fig. 4.2a shows the falls in skeletal muscle rubidium concentrations in both SHRs (■) and WKY rats (□) after the withdrawal of RbCl. There was a greater rate of fall of muscle rubidium in the SHRs compared with the rate of fall of muscle rubidium in the WKY rats. Both sets of data fitted single exponentials, and Fig. 4.2b shows a semilog plot of these

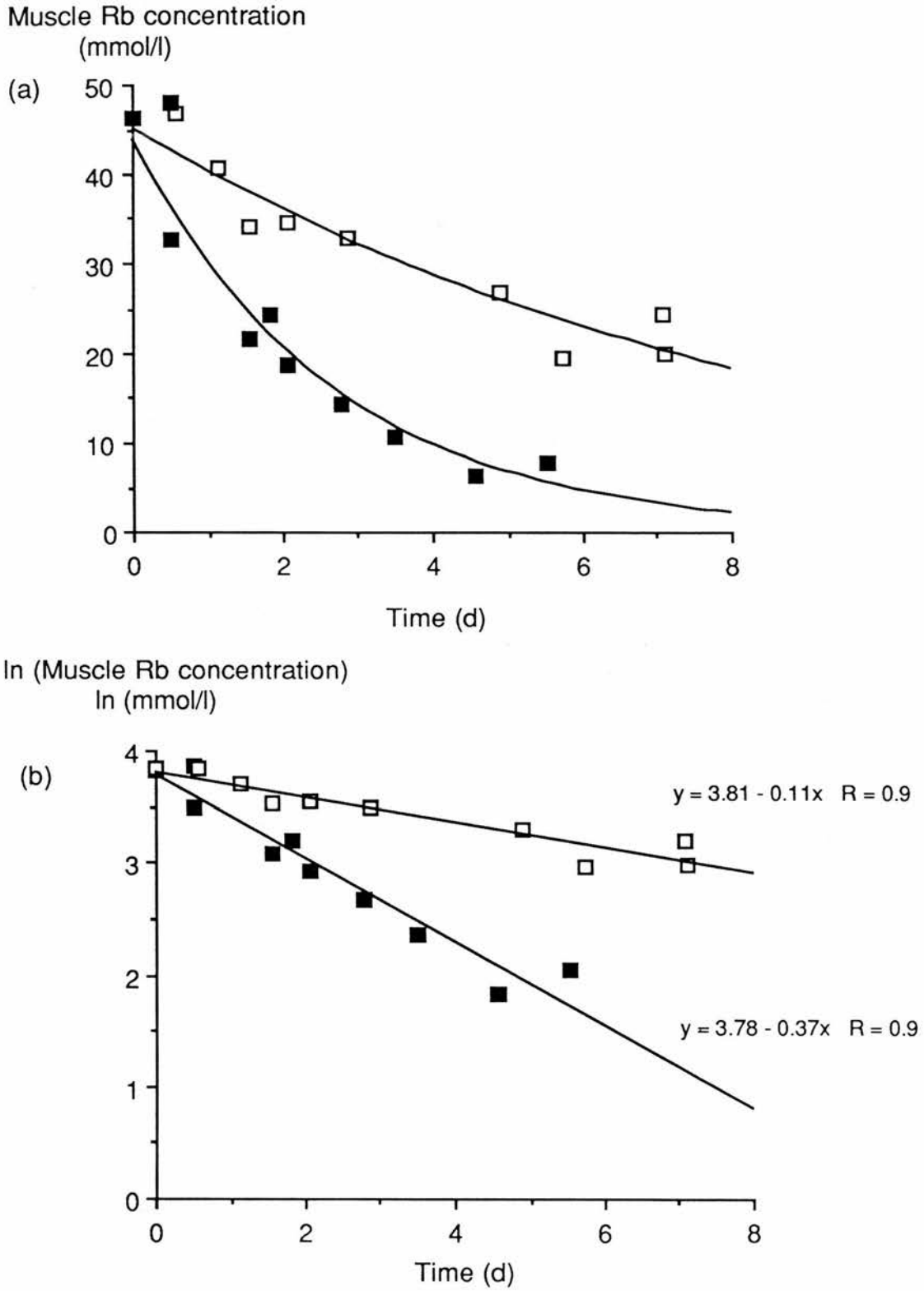


Fig. 4.2 (a) Skeletal muscle rubidium concentrations measured by ^{87}Rb-NMR in SHRs (■) and WKY rats (□). (b) Semilog plot of data shown in (a).

from which the elimination rate constants (λ_{muscle}) and half-lives ($t_{1/2\ muscle}$) of rubidium in the muscle have been calculated by least-squares linearization. The data are shown in Table 4.1. There was a threefold difference between the values in the SHRs and those in the WKY rats ($P < 0.001$).

The single exponential fit to the efflux data suggests that the disposition of rubidium can be adequately modelled by a single compartment. Thus, the elimination rate constant can be used to produce a predicted curve for the increase in muscle rubidium concentrations during loading with RbCl from the following equation:

$$C(t) = C_{ss\ (muscle)} \times (1 - e^{-\lambda musclet}) \qquad (1)$$

where $C_{ss\ (muscle)}$ is the steady-state concentration of rubidium in muscle and C(t) is the concentration of rubidium in skeletal muscle at any time (t) during loading with RbCl. These predicted curves are shown in Fig. 4.1 overlying the data points. The rises in rubidium concentrations in skeletal muscle predicted from the elimination rate constants were very close to the measured values in both groups of rats.

There was a greater rate of fall of both erythrocytic and plasma rubidium in the SHRs compared with the rate of fall of erythrocytic and plasma rubidium in the WKY rats. Both sets of data fitted single exponentials, and Fig. 4.3 shows a semilog plot of the rubidium concentrations in plasma and erythrocytes in both SHRs and WKY rats plotted against time in days after the last injection of RbCl. The

Table 4.1. Kinetic parameters calculated from the measured fall in skeletal muscle, plasma and erythrocyte rubidium concentrations after the withdrawal of RbCl following steady state

Rat	Elimination rate constants (λ) (d^{-1})			Half-lifes ($t_{1/2}$) of Rb (d)			Total Clearance of Rb ($L\ kg^{-1}d^{-1}$)	Apparent Volume of Distribution ($L\ kg^{-1}$)
	λ muscle	λ RBC	λ plasma	$t_{1/2}$ muscle	$t_{1/2}$ RBC	$t_{1/2}$ plasma	CL	Vz
SHR	0.37	0.18	0.10	1.90	3.90	6.90	3.33	33.3
WKY	0.11	0.06	0.06	6.30	11.60	11.60	1.81	30.2

elimination rate constants and half-lives of Rubidium in the plasma (λ_{plasma} and $t_{1/2\ plasma}$) and in erythrocytes (λ_{RBC} and $t_{1/2\ RBC}$) were calculated by least-squares linearization. The data are shown in Table 4.1. There was a threefold difference between the values in the SHRs and those in the WKY rats for erythrocytes and a twofold difference for plasma ($P < 0.001$).

Total clearance of a substance (CL) is given by the equation:

$$CL = Dosage/C_{ss\ (plasma)} \qquad (2)$$

where $C_{ss\ (plasma)}$ is the plasma concentration at steady state.
The apparent volume of distribution of a substance (V_z, in litres of plasma) is given by the equation:

$$V_z = CL/\lambda_{z\ plasma} \qquad (3)$$

where $\lambda_{z\ plasma}$ represents the elimination rate constant from plasma.

The daily dosage of rubidium was the same in both groups of rats but there was a smaller steady-state plasma rubidium concentration in the SHRs and so the total clearance of rubidium in the SHRs was greater (see eqn (2)). This increase in clearance accounted for the increase in $\lambda_{z\ plasma}$ in the SHRs, and there was no difference in the apparent volume of distribution of Rubidium in the SHRs (see eqn. (3)) compared to the WKY rats. The calculated values of V_z and CL are given in Table 4.1.

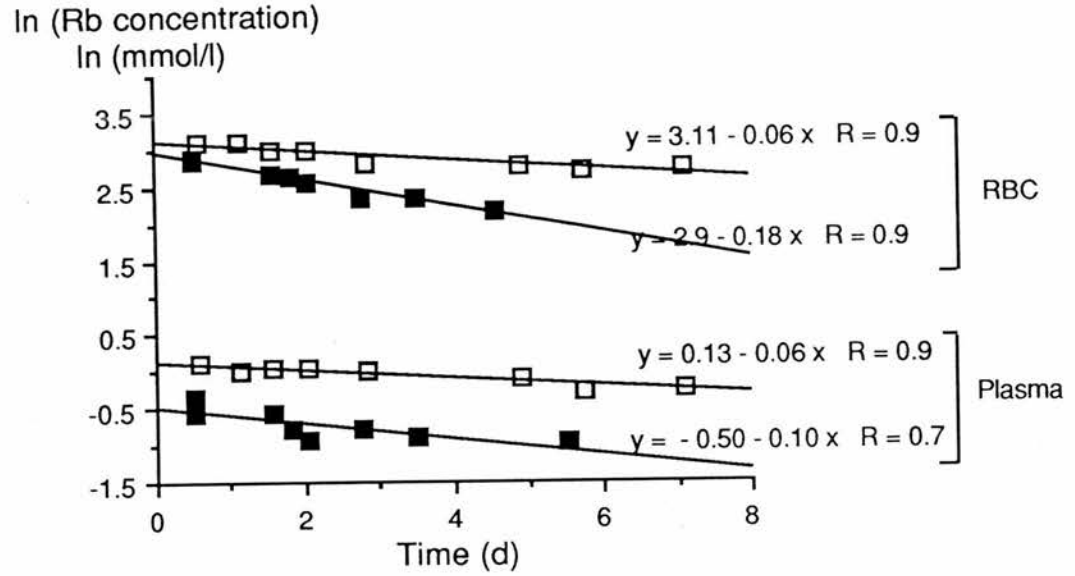


Fig. 4.3 Erythrocytic (RBC) and plasma rubidium concentrations measured by flame atomic absorption spectrophotometry in spontaneously hypertensive rats (■) and Wistar-Kyoto rats (□) after withdrawal of RbCl (semilog plot).

4.4 DISCUSSION

In this study whole tissue concentrations of rubidium in rat hindquarter skeletal muscle were measured non-invasively *in vivo* during repeated administration of RbCl and after its withdrawal. It can be calculated that about 98% of the total concentration of rubidium is intracellular. This is based on the assumption that interstitial fluid has the same rubidium concentration as plasma. The steady-state concentrations of rubidium in whole tissue, plasma, and erythrocytes were 45 mmol/l, 1 mmol/l, and 20 mmol/l respectively (Figs. 4.2 and 4.3). Thus, the contribution of erythrocytic rubidium and extracellular (plasma) ru-

bidium to the total tissue rubidium signal is of the order of two per cent. In contrast, both intracellular and extracellular *water* contribute to the NMR proton signal, and so ^{87}Rb-NMR measurements of tissue rubidium concentrations underestimate intracellular rubidium concentrations.

There was a higher rate of accumulation of rubidium concentrations in the skeletal muscle of the SHRs compared with the skeletal muscle of the WKY rats during loading with the same dose of RbCl, in conjunction with a marked increase in the rate of efflux of rubidium from SHR muscle after withdrawal. Since at steady-state muscle rubidium concentrations were the same in SHRs and WKY rats, these findings imply that there was an increase in the rate of rubidium influx into SHR skeletal muscle and that this increase was equal in degree to the increase in the rate of rubidium efflux from the muscle. This interpretation is supported by the finding that the observed time-course of intracellular rubidium accumulation was closely predicted from the elimination rate constant calculated from the data obtained after the withdrawal of RbCl (Fig. 4.2).

A similar difference in the rate of efflux of rubidium from the erythrocytes of the SHRs, in conjunction with the same steady-state intraerythrocytic concentrations in both SHRs and WKY rats, also implies a similar increase in the rate of rubidium influx from erythrocytes in the SHRs.

Over 90% of cellular rubidium uptake occurs via Na^+/K^+-ATPase transport activity in both rat and man (Bernstein and Israel, 1970;

Overbeck et al., 1981). In the rat this has been shown in brain and erythrocytes, and it is likely that it also applies in skeletal muscle. Thus, the degree of increase in the rate of *in vivo* rubidium influx into both skeletal muscle and erythrocytes shown here implies that Na^+/K^+-ATPase transport activity in SHRs is markedly increased *in vivo*.

The threefold increase in the rate of rubidium efflux shown here *in vivo* in both skeletal muscle and erythrocytes is comparable with the threefold increase in the rate of efflux of K^+ which has been shown *in vitro* in erythrocytes obtained from SHRs and from patients with essential hypertension (Orlov et al., 1989).

This increase in the rate of efflux of K^+ from erythrocytes obtained from both essential hypertensive patients and SHRs has been shown to be due to an increased rate of efflux via calcium-activated K^+ channels (Orlov et al., 1989). Since calcium-activated K^+ channels are present in skeletal muscle (Golowasch et al., 1986), it is possible that the increased rubidium efflux from skeletal muscle in SHRs found in this study could also result from increased opening of calcium-activated K^+ channels. However, the rubidium efflux could also be linked to a regulatory volume decrease, in response to a cell volume increase, caused by an increase in the activity of the Na^+/H^+ antiporter in skeletal muscle, as I shall now argue.

As will be shown (see Chapter 5) there is evidence that Na^+/H^+ antiporter activity is increased *in vivo* in the skeletal muscle of SHRs. Thus, this form of hypertension in the rat is associated with increased Na^+/H^+ antiporter activity, increased Na^+/K^+-ATPase activity, and an

increased rate of rubidium efflux (and by implication an increased rate of K^+ efflux).

The major mechanisms whereby cells increase their volume are via increased Na^+/H^+ antiporter activity and increased HCO_3^-/Cl^- exchange (for review Hoffman and Simonsen, 1989). Thus, an increase in Na^+/H^+ antiporter activity, would result in a tendency for the cell to increase in volume, and this would in turn activate the main mechanism for reducing cell volume, namely K^+ and Cl^- effluxes. This may or may not be calcium dependent (Hoffman and Simonsen, 1989). Increased Na^+/H^+ antiporter activity in conjunction with increased K^+ and Cl^- effluxes would increase intracellular Na^+ and decrease intracellular K^+ concentrations, which would in turn increase Na^+/K^+-ATPase activity (Schwartz et al., 1975).

In addition to the differences between SHRs and WKY rats, it will also be noted that there are differences within each group of animals between the values of $t_{1/2\ plasma}$ on the one hand and $t_{1/2\ muscle}$ on the other, the former being longer than the latter (Table 4.1). This implies that the fall in muscle rubidium in both SHRs and WKY rats is determined by a factor other than renal elimination of rubidium, and is evidence that rubidium is redistributed to other tissues of the body from muscle after withdrawal of RbCl. Furthermore, there is *in vitro* evidence that renal Na^+/K^+-ATPase activity is not altered in SHRs (Garg et al., 1985). Although the SHRs had a greater clearance of rubidium than WKY rats (Table 4.1), the faster rate of fall of muscle rubidium concentrations in the SHRs cannot entirely be explained by increased rubidium clearance and is evidence for a faster rate of redis-

tribution of rubidium from muscle after RbCl is withdrawn in SHRs. This faster rate of rubidium redistribution may also be evidence for increased Na^+/K^+-ATPase activity in tissues other than skeletal muscle in the SHRs.

This redistribution of rubidium from skeletal muscle to other tissues suggests that skeletal muscle acts as a store for K^+, so that during either excessive K^+ loss or reduced K^+ intake, plasma K^+ concentrations are maintained at the expense of muscle K^+. This would explain the findings of others, that during K^+ depletion plasma K^+ concentrations are maintained despite marked falls in skeletal muscle K^+ (Heppel, 1939; Nørgaard et al., 1981). The fall in K^+ concentration during K^+ depletion in skeletal muscle has also been shown to occur in vascular smooth muscle (Aalkjær et al., 1985) and so similar mechanisms may control K^+ concentrations in these two tissues. However, in contrast, myocardial K^+ concentration falls only slightly and brain and liver K^+ concentrations are maintained during K^+ depletion (Kjeldsen et al., 1984), which suggests that the mechanisms responsible for controlling K^+ concentrations in these tissues are different.

4.5 CONCLUSIONS

This study has shown that the rates of influx and efflux of rubidium are equally increased in skeletal muscle in SHRs *in vivo* and that the rate of rubidium efflux is increased in their erythrocytes. The increases in the rates of rubidium influx and efflux were balanced, so that there was no difference in the steady-state concentrations of rubidium in either skeletal muscle or erythrocytes. These findings

suggest that in the skeletal muscle and erythrocytes of SHRs there is increased Na^+/K^+-ATPase activity and an increased rate of K^+ efflux. It has also shown that SHRs had a faster redistribution of skeletal muscle rubidium than WKYrats, and this could be explained by increased Na^+/K^+-ATPase activity in tissues other than skeletal muscle and erythrocytes in the SHR.

These findings of increased *in vivo* Na^+/K^+-ATPase activity are against the hypothesis that inhibition of Na^+/K^+-ATPase is responsible for increasing vascular tone in hypertension. It is much more likely that changes in Na^+/K^+-ATPase activity found in association with hypertension are due to altered intracellular Na^+ and K^+ concentrations, which could in turn result from increased Na^+/H^+ antiporter activity and increased K^+ and Cl^- effluxes. This will be discussed later (see Chapter 9).

This work also shows the importance of skeletal muscle in K^+ homoeostasis and in conjunction with other published work it suggests that the mechanisms controlling potassium concentrations in skeletal muscle are different from mechanisms in other tissues, such as the brain and cardiac muscle.

Chapter five

Na^+/H^+ antiporter activity *in vivo* in the spontaneously hypertensive rat

5.1 INTRODUCTION

Increased activity of the Na^+/H^+ antiporter has been found *in vitro* in cells obtained both from patients with essential hypertension (Livne et al., 1987) and from vascular smooth muscle cells of the spontaneously hypertensive rat (Kuriyama and Aviv,1987). In addition, it has been shown that short-term exposure to vasoconstrictors such as angiotensin II and endothelin increases the activity of the Na^+/H^+ antiporter in vascular smooth muscle (Canessa et al., 1988; Richards et al., 1989). However, it is not known whether increased Na^+/H^+ antiporter activity causes, or results from, increased vascular smooth muscle contraction.

The Na^+/H^+ antiporter is one of the most important mechanisms for controlling intracellular pH, and it does this by exchanging extracellular Na^+ ions for intracellular H^+ ions. The properties of the Na^+/H^+ antiporter have been described in detail in several recent reviews (Aronson and Boron, 1986; Grinstein and Rothstein, 1986; Madshus, 1988; Hoffman and Simonsen, 1989; Seifter and Aronson, 1986). Antiporter activity is mainly controlled by changes in intracellular H^+ ion concentrations, cytoplasmic H^+ ions acting as allosteric activators of Na^+/H^+ exchange (Aronson et al., 1982), and the Na^+/H^+ exchange is driven by the combined transmembrane gradients of Na^+ ions and H^+ ions, with a stoichiometry of 1:1. In skeletal muscle the Km of the exchanger is 6.9×10^{-8} M, equivalent to a pH of 7.16 (Vigne et al., 1985). External H^+ ions can also act as non-competitive inhibitors of external Na^+ ions, and can alter antiporter activity within the physiological range of external pH (Vigne et al.,1982). A low intracellular

Na^+ concentration also alters antiporter activity, by causing a shift in the Km of the antiporter towards a more alkaline pH (Grinstein et al., 1984).

A possible link between increased Na^+/H^+ antiporter activity and increased vascular smooth muscle tone in essential hypertension is via interactions with the calcium-activated proteins important for muscle contraction, although these interactions are complex. The actions of several such proteins, including calmodulin, are reduced by increased intracellular acid (Roos and Boron, 1981; Fabiato and Fabiato, 1978; Busa and Nuccitelli, 1984). For example, in vascular smooth muscle calmodulin, when complexed with calcium, regulates the phosphorylation of myosin light-chain kinase, which results in smooth muscle *contraction* (Carafoli, 1987). On the other hand, Ca^{++}-calmodulin also activates Ca^{++}-ATPase, which controls resting cytosolic Ca^{++} and is the main mechanism responsible for vascular smooth muscle *relaxation* (Carafoli, 1988). Any change in cytosolic acid caused by altered Na^+/H^+ antiporter activity will affect both of these actions of calmodulin and the net effect on the force of contraction will depend on the balance achieved between these two mechanisms. It is not clear to what extent these two different mechanisms would be altered by changes in Na^+/H^+ antiporter activity. However, several workers have shown that increased antiporter activity leads to blunting of agonist-mediated Ca^{++} transients and a reduction in the amplitude of Ca^{++}-dependent vascular smooth muscle contraction (Berk et al., 1987b; Galizzi et al., 1987). Thus, it has been proposed that increased Na^+/H^+

antiporter activity and reduced cytosolic acid may by these actions reduce vascular tone rather than increase it (Aviv, 1988).

Previous studies of Na^+/H^+ antiporter activity in hypertension have all been made *in vitro* (Livne et al., 1987; Kuriyama and Aviv, 1987) or *ex vivo* (Izzard and Heagerty, 1989). *In vitro* experiments on skeletal muscle have shown that the Na^+/H^+ antiporter is responsible for about 80% of the pH recovery after acidification (Aickin and Thomas, 1977a). With this knowledge it can be predicted that if there was any significant alteration in Na^+/H^+ antiporter activity in skeletal muscle *in vivo*, it would be detected by an altered cytosolic acid response during contraction. In this study ^{31}phosphorus nuclear magnetic resonance spectroscopy (^{31}P NMR) was used to make *in vivo* measurements of the changes in cytosolic acid concentrations in skeletal muscle during isometric contraction, in both spontaneously hypertensive rats (SHRs) and normotensive Wistar-Kyoto (WKY) rats. Skeletal muscle was studied because of the difficulty of measuring *in vivo* pH changes in vascular smooth muscle using ^{31}P NMR. Furthermore, the change in muscle tension was measured simultaneously in order to examine the relationship between muscle tension development and changes in cytosolic acid. The cytosolic acid response during contraction was also studied with and without amiloride, an inhibitor of the Na^+/H^+ antiporter (L'Allemain et al., 1984).

5.2 METHODS

(see Chapter 2)

5.3 RESULTS AND PARTICULAR DISCUSSION

5.31 Altered acid response during isometric muscle contraction produced by sciatic nerve stimulation

During isometric muscle contraction the rate of rise of cytosolic acid and the maximum cytosolic acid concentration achieved were significantly lower in the SHRs than in the WKY rats. These results are shown in Fig. 5.1a, where they are compared with the results of the same experiment carried out in the presence of amiloride (Fig. 5.1b), as discussed below. The AUC between 0 and 600 s was significantly lower in the SHRs than in the WKY rats (P=0.006) in the absence of amiloride. In both SHRs and WKY rats the acid concentration rose to a maximum after 200 s and then fell to slightly above resting values, remaining at this value despite continued stimulation. The rate of fall of acid was the same for both SHRs and WKY rats.

This difference in the change in cytosolic acid concentration during stimulation could result from one or more of the following mechanisms:

i) an altered rate of acid production;

ii) different cytosolic buffering of acid;

iii) an altered rate of acid efflux.

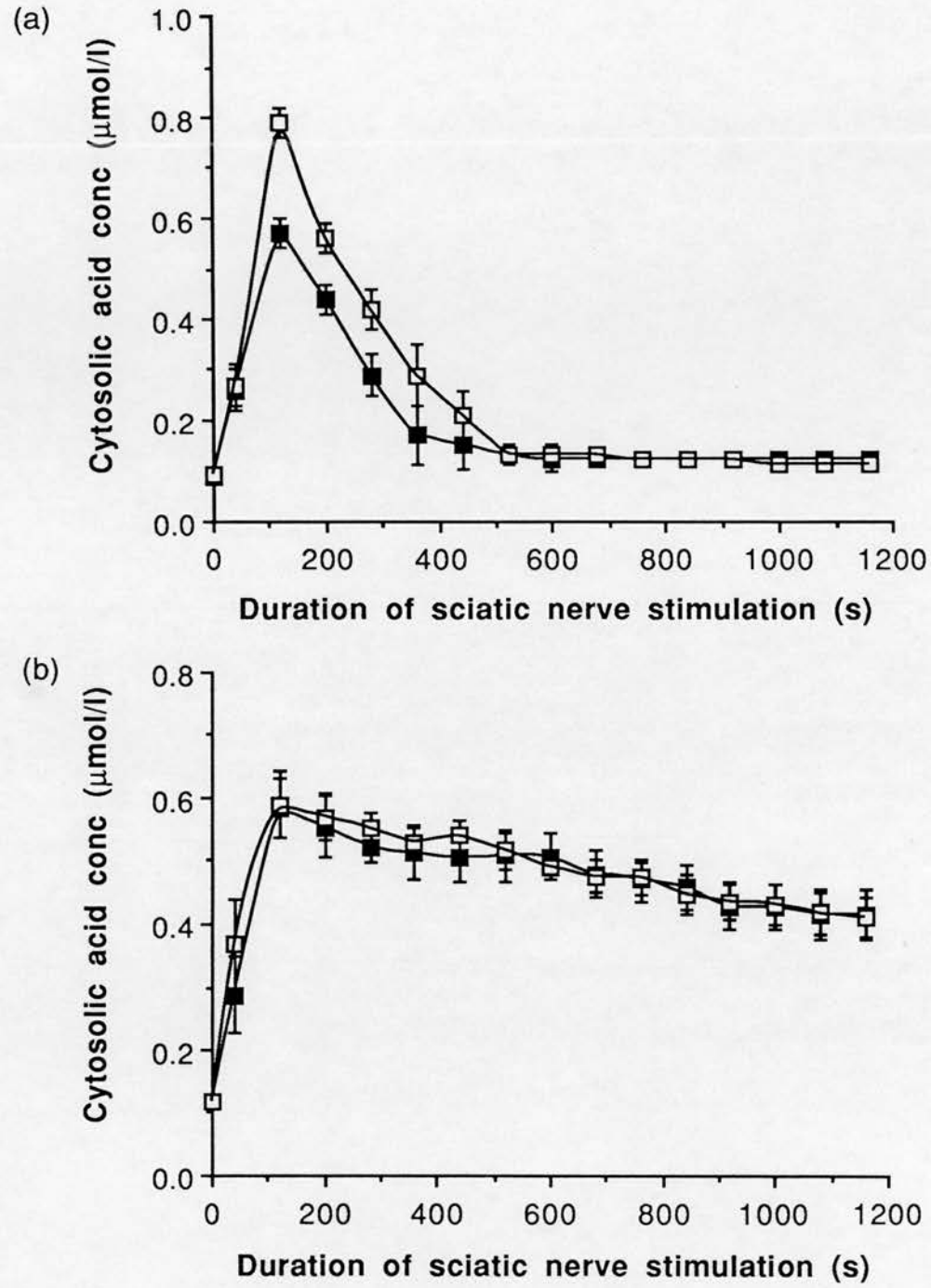


Fig. 5.1 Changes in muscle cytosolic acid during 10 Hz isometric contraction produced by sciatic nerve stimulation in SHRs (■) and WKY rats (□) (a) before (n=6) and (b) after (n=5) the intra-arterial administration of 0.4 mmol/kg amiloride. Bars show SEM.

The possible roles of these different mechanisms were investigated, with the following results.

5.311 *Acid Production*

There were no significant differences between resting concentrations of muscle lactate, blood lactate, or glycogen in the two groups of rats. This was also the case after 2 min of muscle stimulation (Table 5.1). These results imply similar rates of glycogenolysis and lactic acid production during muscle contraction in SHRs and WKY rats.

5.312 *Cell Buffering*

Typical ^{31}P NMR muscle spectra obtained at rest and during stimulation are shown for both SHRs and WKY rats in Figs. 5.2 a and b. They may be compared with the results of the same experiment carried out in the presence of amiloride (Figs. 5.2c and 2d), as discussed below. At rest the inorganic phosphate (P_i) peak was very small and the phosphocreatine (PCr) peak large. During muscle stimulation in both SHRs and WKY rats the PCr peak reached a minimum after 120 s and then started to increase in size again. There was a significant difference in the resting concentrations of PCr in SHR and WKY rat muscle ($P < 0.001$). The reason for this is uncertain. The changes in muscle phosphocreatine concentrations during stimulation are shown in Fig. 5.3.

Table 5.1. Muscle lactate and glycogen at rest and after 120s isometric contraction and blood lactate (µmol/ml whole blood) at rest and after 120s contraction

		Glycogen		Muscle lactate		Blood lactate	
	n	at rest	after 120s	at rest	after 120s	at rest	after 120s
SHRs	6	90 ± 7.4	44.9 ± 3.9	14.3 ± 1.6	64.6 ± 5.9	0.6 ± 0.1	2.1 ± 0.2
WKY rats	6	105.8 ± 11.4	49.1 ± 6.0	13.2 ± 1.5	63.7 ± 3.2	0.6 ± 0.1	1.6 ± 0.3

Means ± SD; n, number of rats; SHRs, spontaneously hypertensive rats; WKY, Wistar-Kyoto.

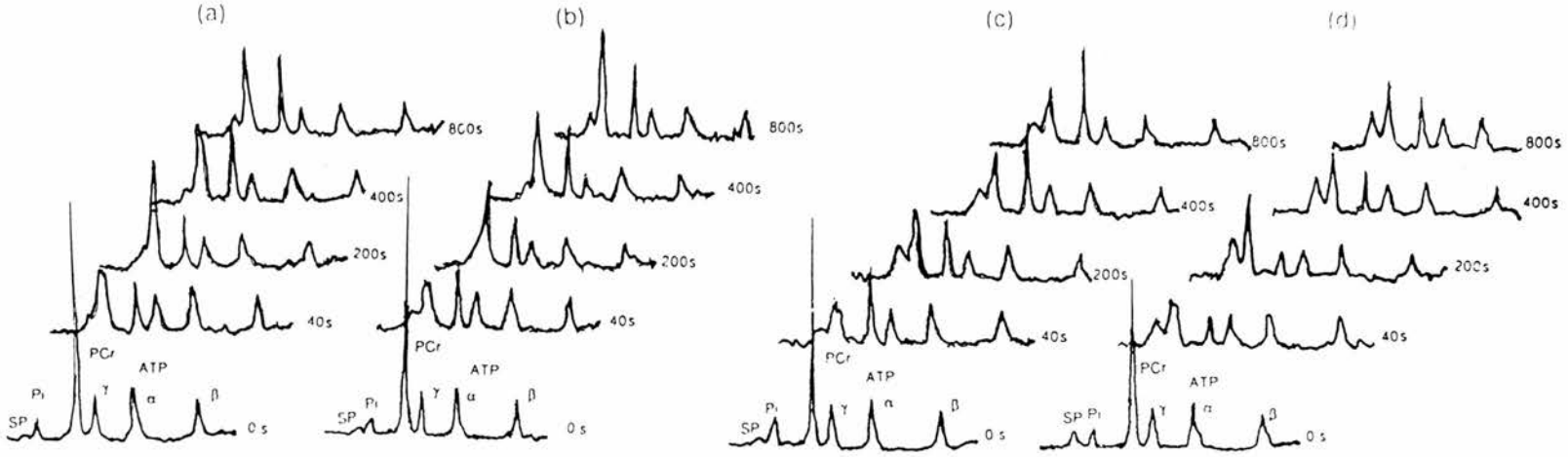


Fig. 5.2 Typical ^{31}P NMR spectra from skeletal muscle in (a) a WKY rat and (b) an SHR after 0, 40, 200, 400, and 800 s of sciatic nerve stimulation in the absence of amiloride and in (c) a WKY rat and (d) an SHR after 0, 40, 200, 400, and 800 s of sciatic nerve stimulation, after the intra-arterial administration of 0.4 mmol/kg amiloride. SP, sugar phosphate; P_i, inorganic phosphate; PCr, phosphocreatine; ATP, adenosine triphosphate.

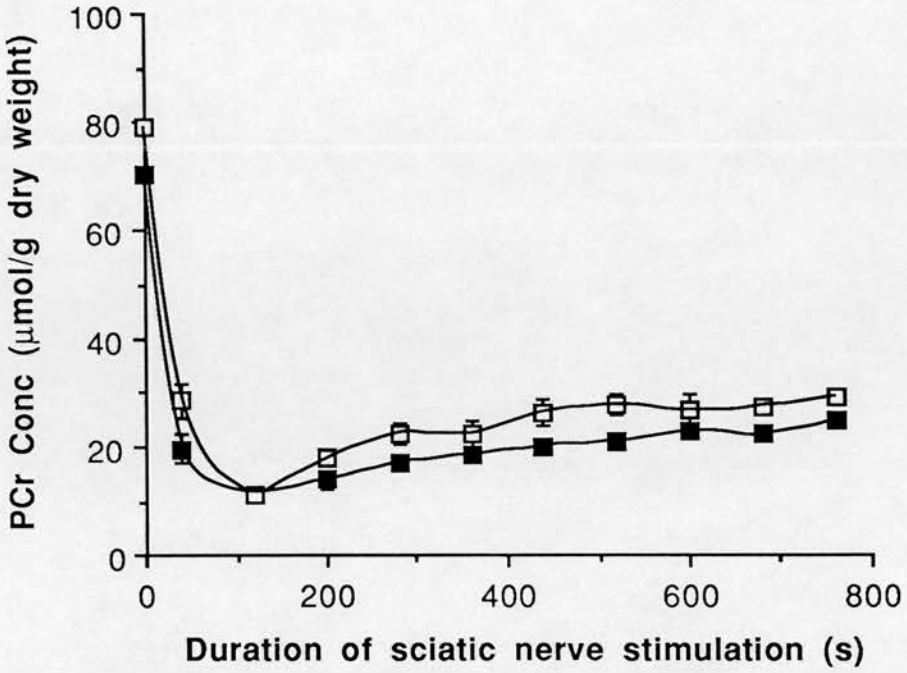


Fig. 5.3 Changes in muscle phosphocreatine (PCr) concentration(μmol/g dry weight) during sciatic nerve stimulation in spontaneously hypertensive rats (■) and Wistar-Kyoto (□) rats. Bars show s.e.m.; n=6).

Although ATP hydrolysis produces H^+ ions, PCr breakdown occurs at a faster rate than ATP hydrolysis, and as this process uses up H^+ ions the net effect is a reduction in the overall rise in intracellular acid during contraction. Therefore, in skeletal muscle PCr acts as one of the most important intracellular buffers in skeletal muscle (Wolfe et al., 1988). From Fig. 5.3 it can be seen that there was a similar fall in PCr during muscle contraction in the SHRs and the WKY rats. The rate of ATP utilization was also the same in the two groups of rats: the similarity in lactate production in the SHRs and WKY rats implies that ATP production was the same, since these muscles are glycolytic

(Armstrong and Phelps, 1984); the similarity in the rate of fall in skeletal muscle ATP concentrations therefore, during contraction in the SHRs and the WKY rats (data not shown), then implies that the rate of ATP utilization was also the same. In addition, the changes in the concentrations in P_i were also the same between SHRs and WKY rats which suggests that differences in buffering from P_i were not different in the two groups.

Thus, these results imply that a difference in cytosolic buffering from changes in PCr and P_i would not explain the difference in acid rise between SHRs and WKY rats during muscle contraction.

An alternative mechanism for the buffering of cytosolic contents is via bicarbonate influx. However, the effects of amiloride on the acid responses in SHR and WKY rat muscle, described below, suggest that the difference in acid response in contracting skeletal muscle between SHRs and WKY rats is not due to this mechanism.

The possible involvement of other cellular buffers is unlikely, since together PCr, P_i and bicarbonate account for almost all sarcoplasmic buffering (Wolfe et al., 1988).

5.313 *Acid Efflux*

The blood lactate concentrations at rest and after stimulation for 2 min in both groups of rats are shown in Table 5.1. Acid efflux from

skeletal muscle cells can occur either from efflux of lactate or by H^+ ion efflux via Na^+/H^+ exchange. Lactate efflux from muscle has been shown to occur via two mechanisms, passively down its concentration gradient, and also in exchange for extracellular hydroxyl ions via a lactate monocarboxylate exchanger (Juel, 1988). If one assumes that the uptake of lactate by the liver is the same in the WKY rats and the SHRs, the lack of difference between the blood lactate concentrations in the SHRs and WKY rats both at rest and during contraction implies that the rates of lactate efflux from the skeletal muscle are the same in both SHRs and WKY rats.

5.31 The effects of amiloride

The above results suggest that the reduction in acid response in the SHRs is due either to an increase in the activity of the Na^+/H^+ antiporter, or to an increase in bicarbonate influx. Bicarbonate influx has been shown to account for about 20% of the acid recovery after muscle acidification with ammonium chloride (Aickin and Thomas, 1977a), but in a recent study the recovery of cytosolic pH after muscle contraction was shown to be entirely due to the Na^+/H^+ antiporter activity and lactate efflux (Juel, 1988). Nevertheless, in order to investigate the possibility that increased bicarbonate influx, rather than increased Na^+/H^+ antiporter activity, was the cause of the difference in acid response between SHRs and WKY rats, the effects of the Na^+/H^+ antiporter inhibitor amiloride was studied on the acid responses in SHRs and WKY rats, and the results are shown in Fig. 5.1b, for

comparison with the similar experiment carried out in the absence of amiloride.

In both SHRs and WKY rats amiloride caused a significant shift in the pH at rest to a mean of 6.93 SD (0.07) for WKY rats and 6.93 (SD 0.04) for SHRs, compared with a mean of 7.05, SD (0.02) for SHRs and 7.04, SD(0.03) for WKY rats without amiloride ($P < 0.001$). Amiloride also removed the difference in the acid response during contraction between the WKY rats and the SHRs. The *pattern* of acid response was also altered, in that in both groups of animals the acid concentrations failed to return to resting values and remained above 0.4 μM for the duration of the contraction. As discussed below, this result implies that the difference in acid response during sciatic nerve stimulation is due to increased activity of the Na^+/H^+ antiporter in the SHRs.

Despite a dose of amiloride sufficient to inhibit the Na^+/H^+ antiporter completely, the maximum cytosolic acid concentration reached in the presence of amiloride in both groups of rats was less than the maximum acid concentration reached in the WKY rats without amiloride.

Figs. 5.2c and 5.2d show the changes in the ^{31}P NMR spectra obtained during muscle contraction in SHR and WKY rats given 0.4 mmol/kg amiloride, for comparison with the spectra obtained in the absence of amiloride (Figs. 5.2a and 5.2b). The sugar phosphate peak increased markedly during the first 200 s, as in the animals not pretreated with amiloride. However, in the presence of amiloride the sugar phosphate

peak did not decrease in size after 200 s of sciatic nerve stimulation, and persisted throughout the period of contraction. This persistent increase in sugar phosphate most probably reflects continued inhibition of phosphofructokinase activity by continuously elevated cytosolic acid (Trivedi and Danforth, 1966).

After amiloride, the resting muscle ^{31}P NMR spectra in the WKY rats showed a greater P_i and a smaller PCr peak, the sugar phosphate peak remaining unchanged. In contrast, the resting spectra in the SHRs given amiloride showed no change in the P_i or PCr peaks, but a marked increase in the sugar phosphate peak. These changes in the resting spectra occurred in association with a significant *reduction* in resting muscle glycogen concentration and an *increase* in resting muscle lactate concentration in the SHRs (Table 5.2). This implies increased resting glycogenolysis and glycolysis in SHRs in the presence of amiloride.

5.33 Changes in muscle tension development

From Fig. 5.4 it can be seen that in association with the different response of cytosolic acid during muscle contraction, there was also a marked difference in the pattern of twitch tension response between the SHRs and the WKY rats. The initial muscle tension produced from a single twitch was the same for the SHRs and the WKY rats, and ranged between 3 and 4 Newtons (N). However, during isometric contraction the WKY rats developed a mean maximum muscle tension of 7.0 N (SD 0.5), whereas the mean maximum muscle tension

Table 5.2 Muscle lactate and glycogen (μmol/g dry weight) at rest and after 120s isometric contraction and blood lactate (μmol/ml whole blood) at rest and after 120s contraction.(after 0.4 mmol/kg amiloride).

		glycogen		muscle lactate		blood lactate	
	n	at rest	after 120s	at rest	after 120s	at rest	after 120s
SHRs	6	73.8 ± 6.5†	34.4 ± 3.0	22.8 ± 2.1*	63.9 ± 6.8	0.8 ± 0.1	4.8 ± 0.6
WKY rats	6	109.5 ± 1.9	49.1 ± 6.0	12.8 ± 1.2	57.6 ± 3.6	0.8 ± 0.1	3.0 ± 0.6

Means ± SD; n, number of rats.*P = 0.003 versus resting lactate in Wistar-Kyoto (WKY) rats with amiloride, P = 0.001 versus resting lactae in spontaneously hypertensive rats (SHR) and P = 0.002 versus resting lactate in WKY rats without amiloride; †P = 0.001, versus resting muscle glycogen in WKY rats with amiloride.

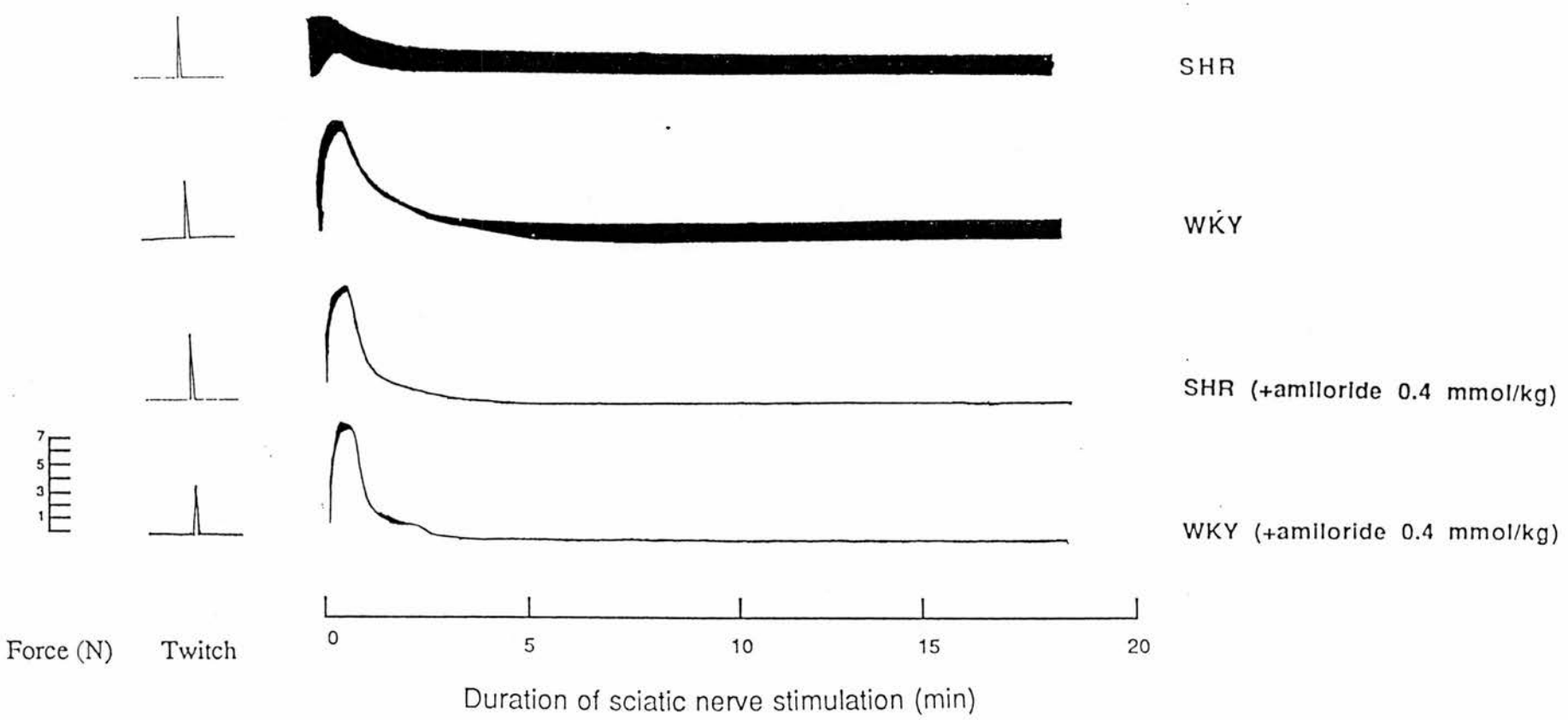


Fig. 5.4 Changes in the force of muscle contraction during sciatic nerve stimulation in skeletal muscle of SHR and WKY rat with and without amiloride (0.4 mmol/kg). N, newtons.

developed by SHRs was only 5.0 N (SD 0.5) ($P < 0.001$), despite a smaller rise in cytosolic acid. There was also a greater relaxation of muscle between twitches in the SHRs. In both SHRs and WKY rats the muscle tension increased to a maximum after 60 s and then fell to a steady level, which was less than 50% of the original twitch tension and was unaffected by the return of cytosolic acid to baseline concentrations. This reduction in muscle tension with continued low-frequency stimulation has been described before, and is a characteristic feature of glycolytic fibres (Edwards et al., 1977).

After amiloride, the maximum muscle tension developed during isometric contraction was 7 N (SD 0.5) in both SHRs and WKY rats, and the relaxation in muscle contraction between twitches also the same, although markedly reduced, as was the steady-state twitch tension.

The difference in the maximum muscle tension achieved between SHRs and WKY rats implies less accumulation of calcium during contraction in the skeletal muscle of the SHRs as compared with the WKY rats, leading to decreased summation of contraction in the SHRs (Luttgau and Stephenson, 1986). This could be due to either reduced release of calcium within the cell in the SHRs or to increased efflux of calcium from the cytosol in the SHRs.

5.4 GENERAL DISCUSSION

5.41 Possible mechanisms of the increase in Na^+/H^+ antiporter activity in SHRs

The finding of increased *in vivo* activity of the Na^+/H^+ antiporter in skeletal muscle could be due either to increased numbers of active antiporters in the muscle cell membrane, i.e. an increase in the maximum velocity of H^+ ion elimination from the cell (V_{max}), or to an increase in the affinity of the antiporter for internal H^+ ions, ie a reduction in the apparent Km of the antiporter, with increased H^+ ion transport at lower H^+ ion concentrations. Form the data obtained in this study it is more likely that the altered antiporter activity in the skeletal muscle of SHRs is due to increased numbers of antiporters, for the following reasons. The amiloride experiment shows that the Na^+/H^+ antiporter is almost entirely responsible for the pH recovery during skeletal muscle contraction *in vivo*, because amiloride inhibited the recovery of acidosis during contraction. At the peak acid concentration during contraction, corresponding to a pH of about 6.0, the antiporter would be working at or near to its Vmax, assuming the Km of the antiporter in skeletal muscle to be 7.16 (Vigne, 1982). As lactate production and efflux were also the same in the two groups of rats, the rate of fall of cytosolic acid after the peak acid concentration was achieved is a good approximation of the rate of H^+ ion efflux via the Na^+/H^+ antiporter. Since the rate of fall of cytosolic acid from maximum was similar in the SHRs and WKY rats, the Vmax of the Na^+/H^+antiporter in skeletal

muscle is likely to be the same in the two groups of animals. Thus, the differences in the rate of rise of cytosolic acid and the peak cytosolic acid concentrations achieved are more likely to be due to increased antiporter activity in the SHRs, resulting from a shift in the Km of the antiporter in skeletal muscle towards a more alkaline pH.

A shift in the Km of the Na^+/H^+ antiporter should lead to an alkaline shift in the resting pH. However, although the resting pHs in the SHRs were slightly more alkaline, this difference did not reach significance. The reason for this may be that increased Na^+/H^+ antiporter activity at rest *in vivo* would increase the concentration of intracellular HCO_3^- leading to increased HCO_3^-/Cl^- exchange (Aickin and Thomas, 1977a) and increased HCO_3^- efflux. This would mask the change in pH resulting from altered Na^+/H^+ antiporter activity in the SHRs.

Numerous activators of the Na^+/H^+ antiporter have been identified, including serum growth factors, insulin, phorbol esters, diacyl glycerol and vasopressin (Grinstein and Rothstein, 1986). The antiporter can also be stimulated by osmotic cell shrinkage (Hoffmann and Simonsen, 1989), and this has been shown to occur in skeletal muscle (Abercrombie and Roos, 1983). Calcium, by activating calmodulin-dependent protein kinases, also appears to be necessary for the full action of activators of the antiporter in some cells , including vascular smooth muscle (Seifter and Aronson, 1986; Mitsuhashi and Eves, 1988). Increased concentrations of cyclic AMP have been shown to increase Na^+/H^+ antiporter activity in

trout red blood cells, mediating the action of ß-adrenoceptor agonists (Borgese et al., 1987), although in kidney brush border cyclic AMP inhibits the Na^+/H^+ antiporter (Grinstein and Rothstein, 1986).

It has been shown that differentiated skeletal myocytes in culture do not increase the V_{max} or reduce the Km of their Na^+/H^+ antiporter when incubated with either growth factors or phorbol esters, although changes in the V_{max} and Km in response to these agents do occur in undifferentiated myoblasts (Vigne et al., 1985). The cause of the increased Na^+/H^+ antiporter activity *in vivo* in the SHRs is therefore not likely to be due to the action of either growth factors or to changes in protein kinase C, which is activated by phorbol esters.

Which, if any, of the other above mechanisms is responsible for the finding of increased *in vivo* Na^+/H^+ antiporter in the skeletal muscle of SHRs is not clear. However, there are three major mechanisms which should be considered.

5.411 *Cell volume regulation*

It is possible that the increased antiporter activity in the skeletal muscle of the SHRs could have occurred as part of a cell volume regulatory mechanism. It is known that cells can increase their Na^+/H^+ antiporter activity in response to shrinkage (Hoffman and Simonsen, 1989). Such a stimulus could result from increased

potassium efflux, which is the main mechanism of cell volume reduction (Hoffman and Simonsen, 1989). Work was presented in chapter 4 that there is a marked increase in the rate of efflux *in vivo* of rubidium from the skeletal muscle of SHRs given rubidium chloride to steady state. This suggests that there may be increased efflux *in vivo* of potassium from skeletal muscle in SHRs, and this has also been shown in rat erythrocytes (Orlov et al., 1989). Thus, increased antiporter activity in skeletal muscle cells in SHRs may result from a volume regulatory response.

5.412 *Changes in cytosolic calcium concentrations*

Increased Na^+/H^+ antiporter activity in vascular smooth muscle and cardiac muscle has also been shown to be strongly coupled to increased cytosolic calcium (Seifter and Aronson, 1986; Hatori et al., 1987). Although calcium does not directly stimulate the Na^+/H^+ antiporter in vascular smooth muscle it appears to be necessary for the action of both protein kinase C-dependent and protein kinase C-independent activation of the antiport (Mitsuhashi and Eves, 1988). The cytosolic Ca^{++} concentration has been shown to be increased in several tissues in the SHR, including vascular smooth muscle (Speiker et al., 1988; Furspan and Bohr, 1986; Bruschi et al., 1985). If the resting concentration of calcium is increased in skeletal muscle cells in SHRs, this could be another explanation of the increased Na^+/H^+ antiporter activity reported here.

5.413 *Altered sympathetic nervous system activity*

It has been shown by several workers that established hypertension in the SHR is associated with increased sympathetic nervous system activity (Falkow et al., 1972; Lee et al., 1986), which would result in stimulation of ß-adrenoceptors in skeletal muscle. This would in turn increase concentrations of cyclic AMP, which could then increase skeletal muscle Na^+/H^+ antiporter activity in the SHR. This could occur either through a direct activation of the antiporter by cyclic AMP, as has been proposed for trout erythrocytes (Borgese et al., 1987), or via increased calcium influx through the action of cyclic AMP at potential-operated calcium channels in the skeletal muscle sarcolemma (Carafoli, 1987). A catecholamine-mediated increase in Na^+/H^+ antiporter activity would also be consistent with the finding in both skeletal muscle and cardiac muscle that catecholamine release accompanying extracellular acidosis attenuates the change in cytosolic pH in these tissues (Clancy et al., 1976).

5.42 The effects of amiloride on skeletal muscle metabolism in the SHR

From this work there is evidence of increased glycogen breakdown and increased glycolysis in the skeletal muscle of SHRs after a dose of amiloride. Glycogen breakdown is stimulated by increases in cyclic AMP, Ca^{++}, and P_i (Rennie and Edwards, 1981). As can be seen from the resting spectra in Figs. 5.2c and 5.2d, the inorganic phosphate peak was smaller in the SHRs compared with the WKY rats, making

increased availability of P_i an unlikely cause of the increased glycogenolysis in SHRs under these conditions. It has also been shown that amiloride in high concentrations produces a dose-dependent reversible inhibition of the catalytic unit of adenylate cyclase, thus reducing cellular concentrations of cyclic AMP (Mahé et al., 1985). Some inhibition of adenylate cyclase would be expected at the dose of amiloride used in this study, which makes increased concentrations of cAMP an unlikely cause of the increased glycogenolysis. This leaves the interesting possibility that the increase in glycogenolysis at rest in the SHRs in the presence of amiloride occurred as a result of *increased* cytosolic calcium, and provides a link between altered calcium metabolism and increased Na^+/H^+ antiporter activity.

5.43 The relationship between Na^+/H^+ antiporter activity and muscle relaxation in both skeletal and vascular smooth muscle following contraction

In a recent study comparing the contractile responses of subcutaneous arteries obtained from patients with essential hypertension and from normotensive controls, blood vessels obtained from patients with essential hypertension showed a marked increase in the speed of relaxation following contraction (Aalkjær et al., 1989). This result is consistent with the similar finding in skeletal muscle in this study and suggests that increased relaxation following contraction is a feature of both vascular smooth muscle and skeletal muscle in hypertension. Furthermore, it has been shown that Na^+/H^+ antiporter activity in vascular smooth muscle *in vitro* is increased in SHRs (Kuriyama and

Aviv, 1987) and this is consistent with the finding in skeletal muscle *in vivo* in this study. Thus, increased Na^+/H^+ antiporter activity in both skeletal muscle *in vivo* and vascular smooth muscle *in vitro* is found in association with increased muscle relaxation.

5.44 Possible mechanism for the differences in muscle contractility between SHRs and WKY rats

During contraction of skeletal muscle there are large increases in cytosolic calcium, and the two main mechanisms responsible for the bulk removal of calcium from the cytosol are the sarcolemmal Na^+/Ca^{++} exchanger and Ca^{++}-ATPase activity in the sarcoplasmic reticulum (Carafoli, 1987; Carafoli, 1988). Of the two mechanisms of bulk calcium removal, increased Ca^{++}-ATPase activity is most likely to be responsible for the increase in relaxation of skeletal muscle contraction between twitches seen in the SHRs in this study. This is because Ca^{++}-ATPase activity is the main mechanism by which calcium efflux occurs during muscle contraction (Carafoli, 1988) and increased Na^+/H^+ antiporter activity would, by reducing the sodium gradient, be expected to reduce Na^+/Ca^{++} exchange. An increase in Ca^{++}-ATPase activity could result from altered calmodulin activity secondary to increased Na^+/H^+ antiporter activity. However, further work in this area is necessary to determine whether increased Ca^{++}-ATPase activity is responsible for the increased skeletal and vascular smooth muscle relaxation, and whether these changes result from increased Na^+/H^+ antiporter activity or another mechanism.

The reduction in maximal muscle tension developed in the SHRs can also be explained by increased Ca^{++}-ATPase activity. Although this is also compatible with increased relaxation of the muscle, as discussed above, it does not necessarily imply that there would be less tension in SHRs muscle at lower levels of stimulation. Since it is known that the vascular smooth muscle of SHRs has an increased rate of relaxation and a normal response to agonists *in vitro* (Aalkjær et al., 1989), this implies that hypertension is associated with *increased* activity of endogenous contractile agonists.

5.5 CONCLUSIONS

Na^+/H^+ antiporter activity *in vitro* has been found to be increased in cells, including blood cells and vascular smooth muscle cells, in both SHRs and patients with essential hypertension. It has now been shown that Na^+/H^+ antiporter activity is also increased in skeletal muscle of SHRs and that this increased Na^+/H^+ antiporter activity is present *in vivo*.

There is growing evidence of a strong link between changes in cytosolic calcium and changes in Na^+/H^+ antiporter activity in vascular smooth muscle. From this work there is indirect biochemical evidence that *in vivo* calcium metabolism is altered in the skeletal muscle of SHRs, and this provides another link between altered Na^+/H^+ antiporter activity and altered calcium metabolism. Calcium could be solely responsible for these changes in Na^+/H^+ antiporter activity or

may amplify Na^+/H^+ antiporter activation in response to a given activating stimulus.

It is also clear from this work that increased Na^+/H^+ antiporter activity is found in association with increased relaxation of skeletal muscle following contraction. Increased relaxation of vascular smooth muscle following contraction has also been shown to occur in subcutaneous arterioles obtained from patients with essential hypertension. Although increased Na^+/H^+ antiporter may not be responsible for the increased relaxation of muscle following contraction, these findings are against the hypothesis that increased Na^+/H^+ antiporter activity is responsible for increasing vascular smooth muscle contraction in hypertension.

It is likely, in view of increased vascular relaxation, that the cause of increased vascular tone in hypertension is due to increased activity of endogenous contractile agonists increasing cytosolic calcium and that increased Na^+/H^+ antiporter activity occurs in association with or as a secondary event resulting from these changes in calcium. Indeed, increased Na^+/H^+ antiporter may even be responsible for increasing vascular smooth muscle relaxation.

Chapter six

Control of pH in the skeletal muscle of the spontaneously hypertensive rat *in vivo*

6.1 INTRODUCTION

The control of intracellular pH is essential for numerous cell functions, including cell division (Busa and Nucciteli, 1984; Grinstein et al., 1989). Recently there has been growing interest in the way in which the regulation of intracellular pH may be altered in hypertension (Aalkjær, 1990), and it has been proposed that altered pH regulation may be responsible for vascular hypertrophy in hypertension (Lever, 1986).

The abnormalities in pH regulation that have been reported in association with hypertension include abnormalities of Na^+/H^+ antiporter activity in both spontaneously hypertensive rats (SHRs) and patients with essential hypertension *in vitro* (Feig et al., 1986; Kuriyama and Aviv, 1987; Canessa et al., 1988) and in SHRs *in vivo* (see Chapter 5). In addition, abnormalities of arterial pH, arterial bicarbonate concentrations (Lucas et al., 1987), and bicarbonate handling by the kidney (Lucas et al., 1988) have been demonstrated in young spontaneously hypertensive rats (SHRs).

In Chapter 5 it was shown that there is evidence for increased Na^+/H^+ antiporter activity in the skeletal muscle of SHRs *in vivo*. However, in that study there was no determination of whether or not intrinsic buffering or bicarbonate handling by skeletal muscle were also different in SHRs *in vivo*.

The aim of the work presented in this chapter was therefore to determine whether or not there was altered bicarbonate metabolism or intrinsic buffering in adult SHRs *in vivo*. In order to do this changes in cytosolic acid concentrations in skeletal muscle at rest and during contraction were made using ^{31}Phosphorus nuclear magnetic resonance spectroscopy (^{31}P NMR) after the administration of an inhibitor of bicarbonate/chloride exchange and during variations in the arterial $PaCO_2$ caused by changing the concentrations of inhaled carbon dioxide.

6.2 METHODS

6.21 Animals

13-week old male SHRs and male WKY rats, ranging in weight from 250 g to 320 g, were obtained from Olac Ltd, Bicester, UK. Anaesthesia was induced with pentobarbital sodium (30 mg/kg) and was maintained during each experiment with halothane and nitrous oxide (0.5-1.0% in 1:1 N_2O:O_2) delivered through a face-mask.The flow of inhalational gas was kept constant at 3 l/min. The concentration of inhaled carbon dioxide was varied to give carbon dioxide concentrations of 0%, 6%, 14%, and 20%, balanced with nitrogen. The inhaled carbon dioxide content was measured using mass spectrometry (VG Mediflex Mass Spectrometer).

Mean arterial blood pressure was monitored by an indwelling catheter in the external carotid artery. It was 125 (SD 8) mmHg in the WKY

rats (n = 20) and 180 (SD 10) mmHg in the SHR (n = 20). There was no significant change in the mean arterial pressure during increased carbon dioxide inhalation.

The rats were prepared for stimulation of the plantaris/gastrocnemius/soleus muscle complex as previously described (see Chapter 2). Sciatic nerve stimulation was performed on both SHRs and WKY rats during inhalation of 20% carbon dioxide, after allowing sufficient time for equilibration of carbon dioxide between the blood and skeletal muscle. The time for equilibration was taken as the time required for the intracellular pH to reach a constant value after changing the carbon dioxide concentration of the inhaled gas. Further groups of SHRs and WKY rats were given 0.2 mmol/kg of 4,4´-di-isothiocyanostilbene-2,2´-disulphonate (DIDS) (Sigma Chemic -al Company Ltd, Dorset, England) 30 min before stimulation. After DIDS the mean arterial pressure fell to 100 (SD 10; n=6) mmHg in the WKY rats and 160 (SD 10; n=6) mmHg in the SHR.

6.22 Monitoring of arterial blood gases

Due to technical problems it was impractical to obtain arterial blood from the animals whilst they were in the magnet, and so separate groups of animals were subjected to identical protocols outside the magnet. Samples of arterial blood (0.25 ml) were taken from the carotid artery catheter at different times and the blood was analysed immediately using a Radiometer ABL330 blood gas analyser, for pH, PaO_2, and $PaCO_2$. A maximum of 0.75 ml of blood was removed

from each animal and this did not cause a change in mean arterial pressure. The plasma bicarbonate concentration was calculated from measured arterial pH and arterial $PaCO_2$ with the following modification of the Henderson-Hasselbalch equation:

$$[HCO^-_3] = \delta \times PCO_2 \times 10^{(pH-pK)} \quad (1)$$

In this formula the CO_2 solubility coefficient (δ) was taken as 0.0298 (Harned and Bonner, 1945) and the dissociation constant (pK) was taken as 6.13 (Hastings and Sendroy, 1925)

6.23 NMR spectroscopy

Spectral parameters have been described previously (see Chapter 2). A single resting spectrum was obtained using a tip angle of 90° at 0% inhaled carbon dioxide and further resting spectra were obtained at 6%, 14%, and 20% inhaled carbon dioxide using a tip angle of 60°. Spectra obtained during muscle contraction were all obtained using a tip angle of 60°. Spectra were quantified by computer line-fitting, and the pH was determined from the chemical shift of inorganic phosphate (P_i) from phosphocreatine (PCr), which was nominally given a shift of 0 p.p.m. (Moon and Richards, 1973). The acquisition trigger of the spectrometer was gated such that 32 spectra were collected every 80 s. The pH measurements thus obtained represented the average muscle pH during sequential 80 s intervals of muscle contraction.

6.24 Measurements of metabolite concentrations

The mean wet/dry weight ratio in muscle specimens was 4.4 (SD 0.2) both at rest and after stimulation for 2 min. There were no statistically significant differences in the mean wet/dry weight ratios during 20% carbon dioxide inhalation or after DIDS.

6.25 Calculation of intracellular bicarbonate concentrations

In calculating intracellular bicarbonate concentrations two assumptions were made. Firstly, that when arterial $PaCO_2$ is at steady state intracellular$PaCO_2$ is also at steady state and is equivalent to arterial $PaCO_2$; this is based on the fact that carbon dioxide has a high lipid solubility, and so one would expect fast equilibration across cell membranes. Second, that the dissociation constant for carbonic acid (pK) in arterial blood is the same as the pK of carbonic acid inside the cell; this is based on the fact that the pK of carbonic acid in any solution is dependent on the absolute temperature and ionic strength of the solution (Putman, 1988) and the fact that both the temperature and ionic strength are likely to be the same in both arterial blood and muscle cells.

Given these two assumptions it is possible to calculate the intracellular bicarbonate concentration from a single measurement of intracellular pH using the values of the steady-state arterial $PaCO_2$ and the pK for arterial blood in eqn (1). Thus, for the different inhaled

carbon dioxide concentrations, it was possible to calculate intracellular bicarbonate concentrations from the intracellular pH, measured using ^{31}P NMR, and the steady-state $PaCO_2$ measured in arterial blood.

6.26 Calculation of buffering capacity in arterial blood and skeletal muscle

Buffering capacity (β) is defined as the number of moles of hydrogen ions ([H^+ ions]) required to reduce intracellular pH (pH_i) by one unit (Van Slyke, 1922). It can be expressed by the following equation:

$$\beta = - \Delta [H^+] / \Delta pH_i \qquad (2)$$

6.261 *Buffering capacity at rest*

Total buffering (β_t) in the muscle cell at rest consists of both bicarbonate buffering (β_{bicarb}) and intrinsic buffering (β_{int}).

When the intracellular $PaCO_2$ is increased the hydrogen ion load to the cell is also increased, and the change in intracellular pH as a result of this hydrogen ion load is determined by β_{int}. As the increase in hydrogen ion load to the muscle cell following an increase in intracellular $PaCO_2$ is also equivalent to the increase in calculated bicarbonate concentration, the β_{int} of the muscle cell can be calculated from a plot of the changes in calculated bicarbonate

concentration against the changes in intracellular pH resulting from an increase in intracellular $PaCO_2$, as shown in eqn (3).

$$\beta_{int} = -\Delta \text{ [calculated bicarbonate]}/ \Delta pH_i \qquad (3)$$

The same method can be used to calculate β_{int} for arterial blood. In this study β_{int} was calculated for both SHRs and WKY rats for both arterial blood and muscle. β_{int} was calculated from the slope of the line, fitted by least squares linearization, relating the changes in calculated mean intracellular and extracellular bicarbonate concentrations to the changes in the measured mean intracellular and extracellular pH respectively at different steady-state values of PCO_2 (Aickin, 1977).

When the $PaCO_2$ is constant the contribution of bicarbonate to buffering is also constant, and β_{bicarb} depends on the resting concentration of intracellular bicarbonate (Putman, 1988), as shown in eqn (4)

$$\beta_{bicarb} = \ln 10 \, [HCO^-_3] \qquad (4)$$

6.262 *Buffering capacity during isometric contraction*

During contraction there is a further contribution to muscle cell buffering from phosphocreatine and phosphate (Wolfe et al., 1988). This was assessed by measuring the changes in phosphocreatine and phosphate concentrations by ^{31}P NMR. during muscle contraction.

6.27 Statistical and mathematical methods

The data are presented as means (SD). Tests of normality were performed on all data (Royston, 1983) and one-way ANOVA was used to compare all groups of rats, using Fisher's Protected Least Significant Difference test. Areas under curves (AUCs) were calculated using Simpson's method.

6.3 RESULTS

6.31 The effects of 20% inhaled carbon dioxide and DIDS on changes in cytosolic acid concentrations during muscle contraction

Fig. 6.1 shows the cytosolic acid response to isometric contraction in untreated WKY rats and SHRs as shown in Chapter 5. In untreated rats the cytosolic acid concentration rose to a maximum after 120 s of stimulation and then returned to baseline concentrations, despite continued stimulation. The AUC between 0 and 600 s was significantly lower in the SHRs than in the WKY rats ($P = 0.006$). This difference is removed by amiloride, an inhibitor of the Na^+/H^+ antiporter, which also prevented the return to baseline cytosolic acid concentrations (see Chapter 5).

Fig. 6.2 shows the cytosolic acid response in SHRs and WKY rats during inhalation of 20% carbon dioxide. As in untreated rats, the cytosolic acid concentration rose to a maximum after 120 s of

stimulation and then returned to baseline concentrations, despite continued stimulation. The AUC between 0 and 600 s was again significantly lower in SHRs than in the WKY rats ($P < 0.001$). In addition, the rate of return to baseline concentrations, has been previously shown to be

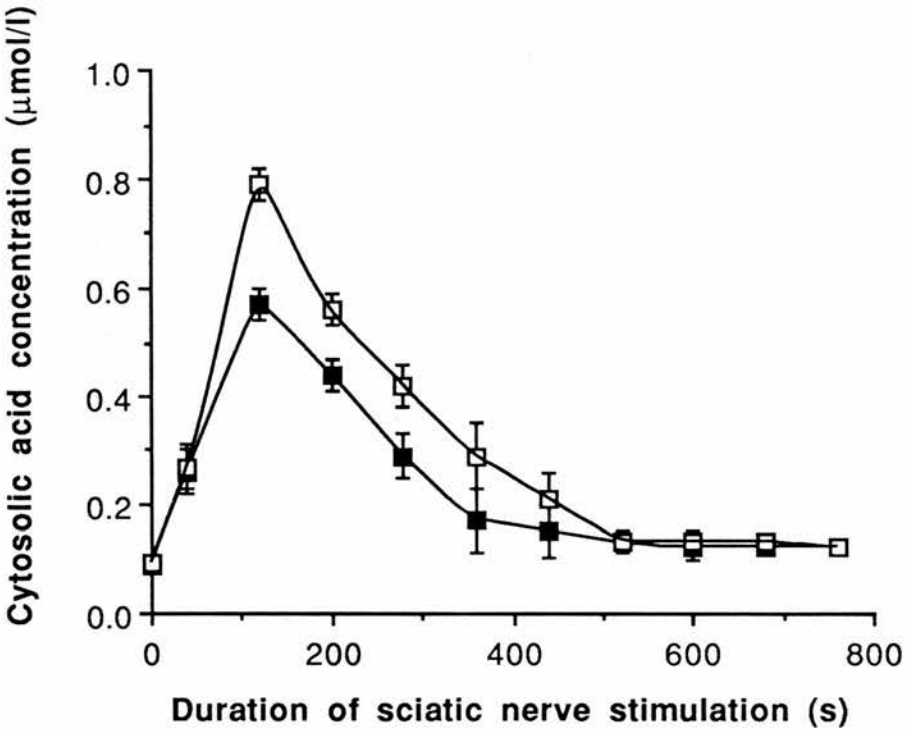


Fig. 6.1. The change in skeletal muscle cytosolic acid concentration (µmol/l) (mean with SEM; n=6) during 10 Hz isometric contraction in untreated WKY rats (□) and untreated SHRs (■) ($P = 0.006$)

abolished by inhibition of Na^+/H^+ antiporter activity (see Chapter 5), was unaltered by a high concentration of inhaled carbon dioxide.

However, there was a significant increase in both groups of animals in both the resting hydrogen ion concentration and the hydrogen ion concentration after 600 s of contraction compared with untreated rats ($P < 0.001$).

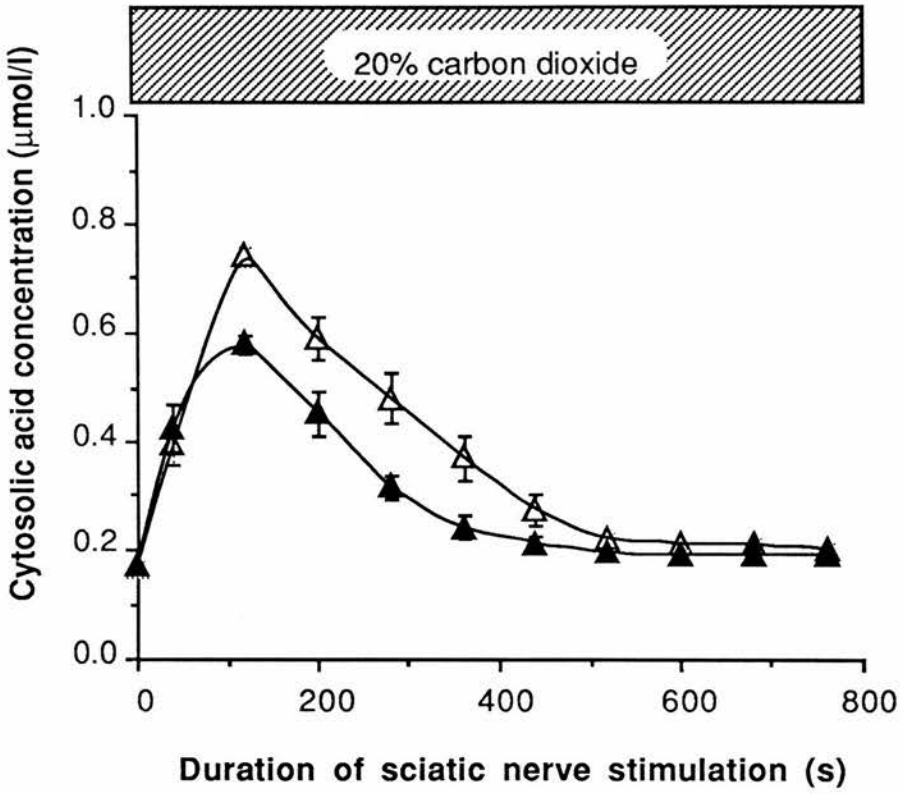


Fig. 6.2. The change in skeletal muscle cytosolic acid concentration (µmol/l) (mean with SEM; n=6) during 10 Hz isometric contraction in WKY rats (Δ) and SHRs (▲) during 20% carbon dioxide ($P < 0.001$).

The administration of 0.2 mmol/kg DIDS before contraction (Fig. 6.3) had no effect on the cytosolic acid response compared to untreated rats. DIDS did not produce a significant alteration in resting cytosolic acid concentrations.

6.32 Changes in concentrations of lactate, phosphocreatine, inorganic phosphate, and ATP during contraction

Table 6.1 shows the concentrations of muscle lactate both at rest and after 120 s of contraction during sciatic nerve stimulation under different experimental conditions. Increased concentrations of inhaled

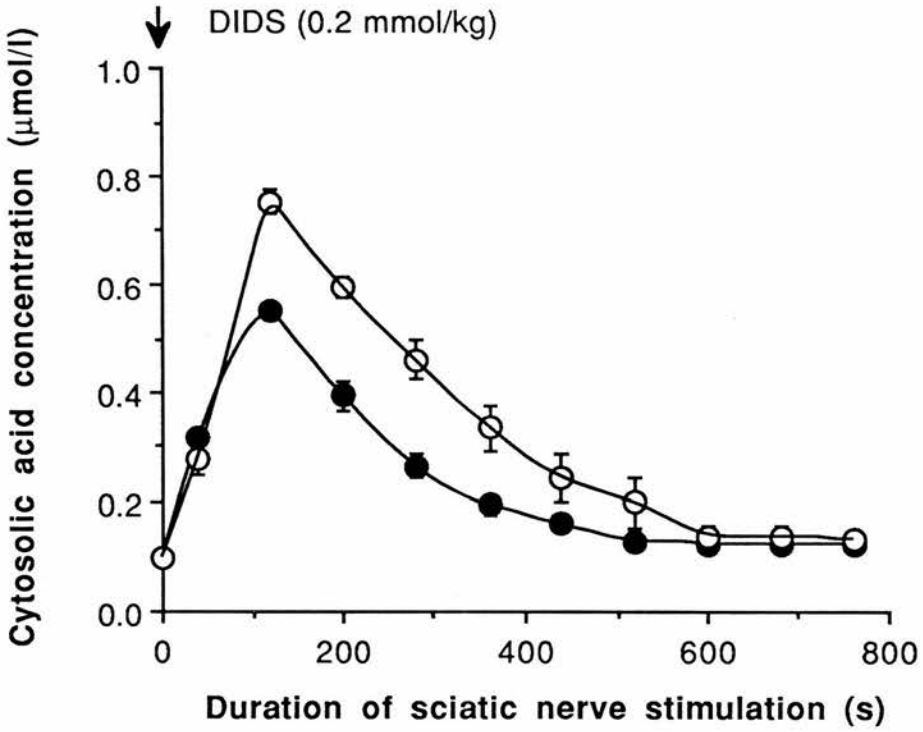


Fig. 6.3. The change in skeletal muscle cytosolic acid concentration (µmol/l) (mean with SEM; n=6) during 10 Hz isometric contraction in WKY rats (o) and SHRs (●) after 0.2 mmol/kg DIDS ($P < 0.001$).

carbon dioxide resulted in a significant reduction in resting lactate concentrations ($P < 0.001$), but DIDS had no effect. In contrast, both

Table 6.1 Muscle lactate concentrations at rest and after 120 s of isometric contraction

		Muscle lactate concentration (μmol/g dry weight)	
Rat	Condition	At rest	After 120 s
WKY	untreated	13.2 (3.7)	63.7 (7.9)
SHR	untreated	14.3 (3.9)	64.6 (14.5)
WKY	20% CO_2	5.8 (3.4)*	95.7 (14.7)†
SHR	20% CO_2	6.2 (2.6)*	103.4 (24.2)†
WKY	DIDS	12.5 (1.5)	97.1 (17.7)†
SHR	DIDS	11.0 (3.5)	105.3 (7.3)†

Values are shown as mean (SD); (n=6)

*P<0.001 compared with all unmarked values at 0 s (ANOVA using Fisher's Protected Least Significant Difference test)

†P<0.001 compared with all unmarked values at 120 s (ANOVA using Fisher's Protected Least Significant Difference test).

20% carbon dioxide and DIDS resulted in similar and significant increases in muscle lactate concentrations at 120 s of contraction ($P < 0.001$). These increases in muscle lactate concentration were not significantly different between SHRs and WKY rats.

Fig. 6.4 shows that there was no significant difference in both the reduction and recovery of PCr, one of the most important buffers in skeletal muscle cytosol (Wolfe et al., 1988), during sciatic nerve stimulation in all groups of animals. Nor was there a significant difference in the change in phosphate concentrations or in the fall in ATP concentrations during contraction in the different groups of animals (data not shown).

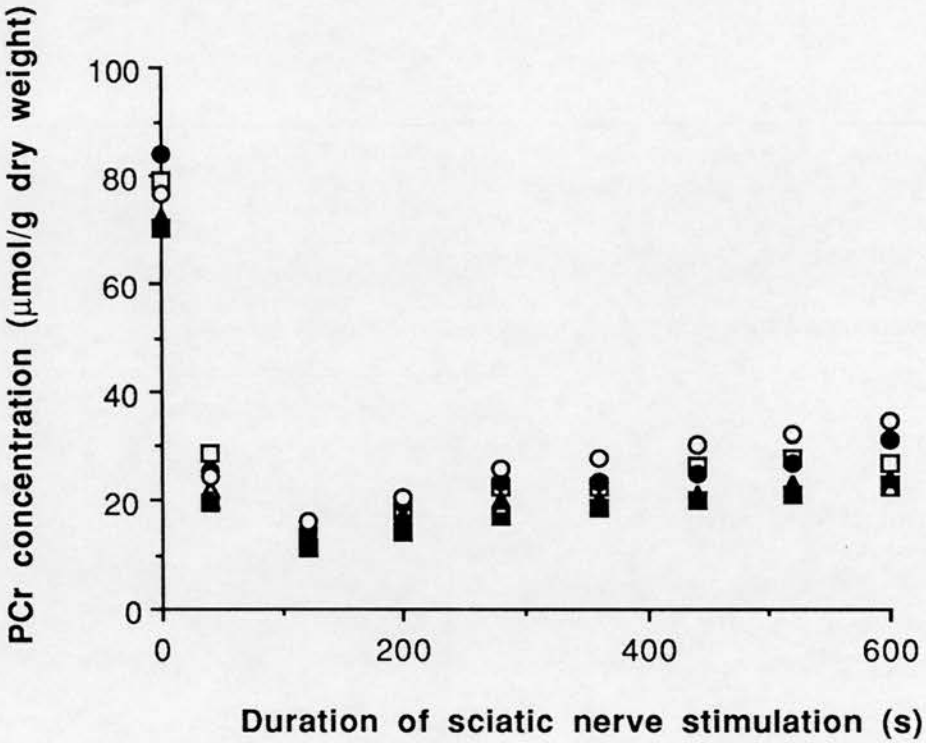


Fig. 6.4. The changes in skeletal muscle phosphocreatine (PCr) concentrations (µmol/g dry weight) during 10 Hz isometric contraction in untreated WKY rats (□) and SHRs (■), during 20% carbon dioxide in WKY rats (△), and SHRs (▲), and after 0.2 mmol/kg DIDS in WKY rats (○), and SHRs (●). For clarity only the mean values are shown.

6.33 Differences in bicarbonate concentrations and intrinsic buffering between SHRs and WKY rats

Fig. 6.5 shows a plot of the mean arterial PCO_2 against the mean arterial and mean intracellular values of pH for both SHRs and WKY rats. At increasing values of PCO_2 there were significant falls in both intracellular pH and arterial pH. However, there was no significant difference in these responses between SHRs and WKY rats.

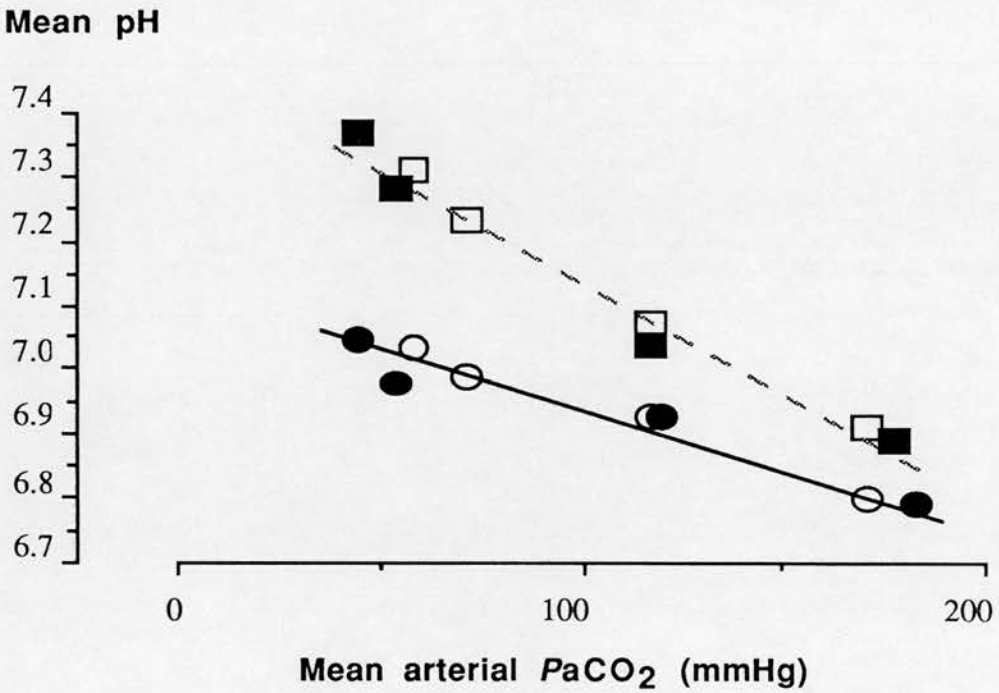


Fig. 6.5. The changes in arterial pH (dotted line) in SHRs (■- - -■) and WKY rats (□- - -□) and the changes in intracellular pH (solid line) in skeletal muscle of SHRs (●——●) and WKY rats (○——○) for a given increase in arterial PCO_2 (Only mean values are shown)

Fig. 6.6 shows the changes in calculated intracellular and extracellular bicarbonate concentrations plotted against the changes in mean values of intracellular and extracellular pH for different concentrations of inhaled carbon dioxide in both SHRs and WKY rats. The intrinsic buffering capacity calculated from these results was 55.5 mmol/l/pH unit for SHRs and 47.6 mmol/l/pH unit for WKY rats. This difference in intrinsic buffering was not significant.

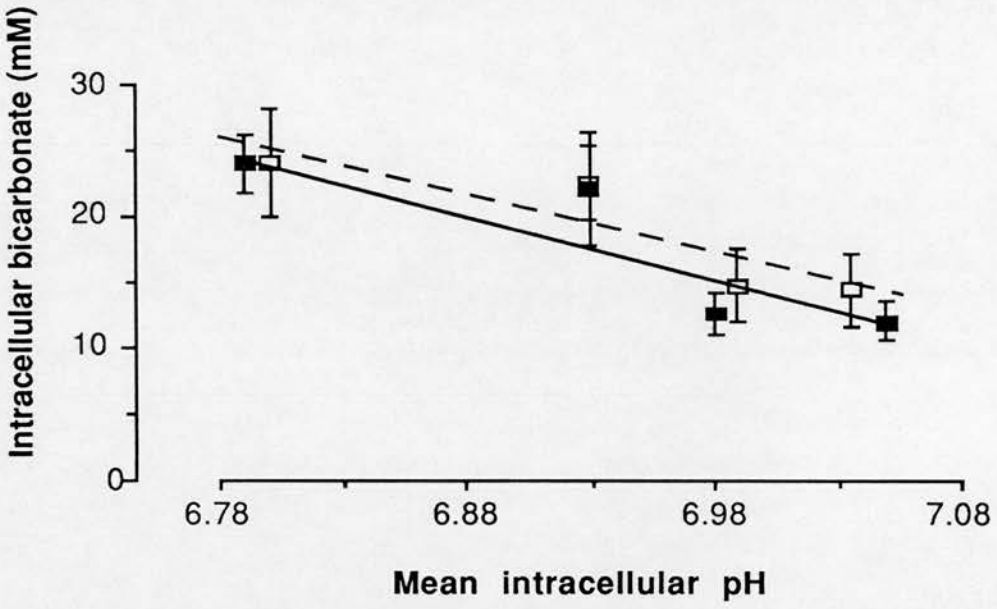


Fig. 6.6. The changes in intracellular bicarbonate in SHRs (■——■) and in WKY rats (□- - -□) for a given increase in arterial PCO_2. Mean values (SD); n=5.

The intracellular bicarbonate concentration in the SHRs at rest was 12.1 (SD 1.6) mmol/l, calculated from a measured intracellular pH of 7.05 (SD 0.02) and an arterial PCO_2 of 44.7 (SD 6.7) mmHg. In WKY rats the intracellular bicarbonate concentration was

14.5 (SD 2.9) mmol/l, calculated from an intracellular pH of 7.04 (SD 0.02) and an arterial PCO_2 of 58.1 (SD 8.9) mmHg. These differences in intracellular pH and bicarbonate concentrations were not statistically significant. There were also no significant differences at rest in the values of arterial pH, $PaCO_2$, PaO_2 and arterial bicarbonate concentrations between SHRs and WKY rats.

6.4 DISCUSSION

In Chapter 5 pH regulation in skeletal muscle at rest and during contraction *in vivo* in SHRs and WKY rats was studied using nuclear magnetic resonance spectroscopy. Using this technique it was shown that there is a significant difference in the cytosolic acid response in skeletal muscle in SHRs compared with WKY rats during 10 Hz isometric contraction, and since this difference was removed by prior administration of amiloride, an inhibitor of the Na^+/H^+ antiporter, this suggested that it was due to increased Na^+/H^+ antiporter activity *in vivo* in SHRs.

However, in this previous study the role of bicarbonate in regulating pH either at rest or during contraction was not determined. Nor was the influence of bicarbonate exchange. Furthermore, amiloride, which was used to define the activity of the Na^+/H^+ antiporter, has actions other than antiporter inhibition (Frelin et al., 1987). In this chapter the experiments described were performed in order to investigate the role of bicarbonate and intrinsic intracellular

buffering in the control of cytosolic pH and to determine whether or not they are altered in hypertension.

In this work two methods were used to determine the contribution of bicarbonate and intrinsic buffering to the control of pH in both resting and contracting skeletal muscle *in vivo* and to determine whether or not there was any evidence for altered bicarbonate handling in skeletal muscle of the SHR. The role of intracellular bicarbonate was studied by increasing the concentration of inhaled carbon dioxide and the role of bicarbonate fluxes by the administration of DIDS, an inhibitor of both sodium-dependent and sodium-independent bicarbonate transport (Aickin, 1986; Kahn et al., 1990).

6.41 The effect of carbon dioxide and DIDS on cytosolic acid concentrations during contraction

Neither carbon dioxide nor DIDS affected the difference in the pattern of cytosolic acid changes during contraction between SHRs and WKY rats. These results show that this difference in the cytosolic acid response during contraction was not due to a difference in bicarbonate exchange in the SHRs. The role of intracellular bicarbonate in the control of resting pH is discussed below.

6.42 Intrinsic buffering in SHRs and WKY rats

The value of intrinsic buffering calculated in this study for skeletal muscle *in vivo* for both SHRs and WKY rats is within the range of

calculated values obtained from the literature for intrinsic buffering of skeletal muscle (Aickin and Thomas, 1977b; Clancy and Brown, 1966; Heisler and Piiper, 1972). No significant difference was found in the intrinsic buffering capacity between SHRs and WKY rats. Since intrinsic buffering also includes resting Na^+/H^+ antiporter activity, this implies that the increase in Na^+/H^+ antiporter activity in SHRs at rest is not sufficient to result in a significant difference in resting intrinsic buffering. This is discussed below in relation to resting skeletal muscle intracellular pH in SHRs and WKY rats.

6.43 The effects of carbon dioxide and DIDS on skeletal muscle metabolism in SHRs and WKY rats

Carbon dioxide and DIDS had no effect on the changes in the concentrations of both phosphocreatine and inorganic phosphate both at rest and during contraction. This is evidence that the intracellular buffering caused by these compounds does not contribute to the difference in cytosolic acid response during contraction between SHRs and WKY rats.

Carbon dioxide caused a significant reduction in muscle lactate concentrations at rest in both SHRs and WKY rats. The reason for this is uncertain, but it could be that the resting acidosis resulting from the carbon dioxide load inhibited phosphofructokinase activity and reduced lactate formation (Trivedi and Danforth, 1966). Both carbon dioxide and DIDS caused a significant increase in muscle lactate

concentrations during contraction, but there was no difference between SHRs and WKY rats.

6.44 The control of resting pH in the skeletal muscle of SHRs and WKY rats

In the previous study (see Chapter 5) no significant difference was found in cytosolic pH in resting skeletal muscle in SHRs compared with WKY rats, despite evidence for increased Na^+/H^+ antiporter activity in the skeletal muscle of the SHRs during contraction. However, in the same study amiloride caused an equal acid shift in the resting pH of the skeletal muscle of SHRs and WKY rats, suggesting that the Na^+/H^+ antiporter is active at rest in skeletal muscle (see Chapter 5). There are three possible reasons for this combination of findings. Firstly, as discussed in Chapter 5, it could be due to increased sodium-independent bicarbonate/chloride exchange, resulting in increased bicarbonate efflux and preventing an alkaline shift in resting pH in the SHRs. Secondly, it could be due to a reduction in intrinsic buffering in the face of increased Na^+/H^+ antiporter activity in SHRs compared with WKY rats. Thirdly, it could be that although Na^+/H^+ antiporter activity is increased in SHRs, at rest this increase in activity is too small to make a detectable difference in resting pH compared with WKY rats.

6.441 *The role of bicarbonate/chloride exchange*

If a change in bicarbonate/chloride exchange were the explanation for the lack of difference in resting values of intracellular pH, inhibition of sodium-independent bicarbonate/chloride exchange by DIDS in the presence of increased Na^+/H^+ antiporter activity in the SHRs should have caused an alkaline pH shift at rest. However, DIDS did not alter resting intracellular pH in either the SHRs or the WKY rats. This result is evidence against the hypothesis that increased sodium-independent bicarbonate/chloride exchange is responsible for the finding of a lack of difference in resting values of intracellular pH between SHRs and WKY rats.

6.442 *The role of intrinsic intracellular buffering*

There was no difference in this study in intrinsic buffering between SHRs and WKY rats. Thus, a reduction in intrinsic buffering in the SHRs cannot be responsible for the lack of difference in resting pH in skeletal muscle between SHRs and WKY rats in the face of increased Na^+/H^+ antiporter activity.

6.443 *The role of the Na^+/H^+ antiporter*

As discussed above intrinsic buffering capacity did not contribute to the control of resting pH. Since intrinsic buffering includes the resting activity of the Na^+/H^+ antiporter this implies that the increase

in Na^+/H^+ antiporter activity in the SHRs is not sufficient to result in a significant difference in resting intrinsic buffering.

To conclude therefore, the most likely reason for the finding that the resting intracellular pH is the same in SHRs and WKY rats is that the increase in Na^+/H^+ antiporter activity in the SHRs compared with WKY rats is too small to make a detectable difference in resting pH. Thus, any small difference in hydrogen ion load to the cell resulting from a small increase in Na^+/H^+ antiporter activity is buffered by intrinsic buffers and does not result in a detectable change in intracellular pH.

However, it should be noted that perturbation of the bicarbonate buffering system by a high concentration of inhaled carbon dioxide altered resting intracellular pH (Fig. 6.2). This is compatible with the *in vitro* finding that bicarbonate/chloride exchange is important in regulating resting pH in skeletal muscle (Aickin, 1986). Since at steady state bicarbonate influx equals bicarbonate efflux DIDS would not be expected to alter net bicarbonate fluxes at rest, assuming that it inhibits all bicarbonate transport systems (Aickin, 1986; Tønnessen et al., 1990; Kahn et al., 1990). Thus, the lack of effect of DIDS on resting intracellular pH (Fig. 6.3) can be explained by the fact that it inhibits both sodium-dependent bicarbonate/chloride exchange (which results in bicarbonate influx) and sodium-independent bicarbonate/chloride exchange (which results in bicarbonate efflux).

6.45 Bicarbonate handling in SHRs and WKY rats

It has been shown by others that in SHRs aged 6 to 12 weeks there are significant reductions in arterial pH and bicarbonate concentrations and that bicarbonate clearance by the kidney is reduced compared with age-matched WKY rats (Lucas et al., 1987; Lucas et al., 1988). However, in this study no significant differences were found between SHRs and WKY rats in regard to arterial pH, arterial bicarbonate concentrations, intracellular bicarbonate concentrations, intracellular pH, or intrinsic buffering. This implies that bicarbonate/chloride exchange at rest is the same in both groups of rats, and therefore it cannot be concluded that bicarbonate metabolism in skeletal muscle in SHRs is different from that in WKY rats.

One possible reason for the difference in the findings presented in this chapter compared with that of previous workers (Lucas et al., 1987; Lucas et al., 1988) could relate to the ages of the rats. In this study 13-week-old adult rats were used whereas Lucas et al. used younger rats (less than 12 weeks old). Thus, alterations in bicarbonate metabolism may be found only in the developing SHRs and not in the adult SHRs with established hypertension.

6.46 The nature of the change in Na^+/H^+ antiporter activity *in vivo* in the skeletal muscle of the SHR

In this study it has been shown that the difference in cytosolic acid response during contraction between SHRs and WKY rats is not due

to a difference in bicarbonate handling, intrinsic buffering, or buffering from phosphocreatine or inorganic phosphate during contraction. Na^+/H^+ antiporter activity is the only other mechanism known to be responsible for pH regulation in muscle during contraction, and this suggests that the difference in cytosolic acid response seen in the presence of a high concentration of inhaled carbon dioxide results from increased hydrogen ion efflux caused by an increase in Na^+/H^+ antiporter activity in SHRs compared with WKY rats. This result substantiates previous findings using amiloride (see Chapter 5).

In Chapter 5 it was argued that the increase in Na^+/H^+ antiporter activity seen in SHRs resulted from an alkaline shift in the apparent Km of the antiporter rather than an increase in its V_{max}. An alkaline shift in the Km of the antiporter would result in a reduced rate of rise of cytosolic acid concentration and a reduced maximum cytosolic acid concentration in SHRs, as demonstrated. At the maximum cytosolic acid concentration achieved, corresponding to a pH of 6.12, the antiporter would be expected to be working at about 92% of its V_{max} if the Km is pH 7.16 in skeletal muscle cells, as has been reported elsewhere (Vigne et al., 1985). Thus, the parallel fall in cytosolic acid concentration to baseline after reaching a maximum in SHRs and WKY rats is evidence that the V_{max} of the Na^+/H^+ antiporter *in vivo* in skeletal muscle of the SHRs and WKY rats is the same.

If the assumption is made that the antiporter is working at the same rate when cytosolic acid concentrations are at a peak in the SHRs as it is in the WKY rats (i.e. 92% of maximum) it can be calculated that the Km of the antiporter in the SHRs is around pH 7.33 *in vivo*. It can also be calculated that at a resting intracellular pH of 7.05 in the SHR the antiporter is 66% saturated. The corresponding value in the WKY rats (resting intracellular pH 7.04) is 57%. In making these calculations the assumption has also been made that there is no change in the co-operativity or stoichiometry of the Na^+/H^+ antiporter (Canessa et al., 1988).

6.5 CONCLUSIONS

In Chapter 5 work was presented which showed that during 10 Hz isometric contraction of rat skeletal muscle there is a characteristic pattern of change in cytosolic acid concentrations *in vivo*. In this chapter further work has been presented which shows that this pattern of cytosolic acid response is unaffected by 20% inhaled carbon dioxide or by 4,4´-di-isothiocyanostilbene-2,2´-disulphonate (DIDS). This is evidence that bicarbonate exchange *in vivo* is not important in the control of cytosolic acid concentrations during skeletal muscle contraction.

This work has also shown that the difference in cytosolic acid response during contraction between SHRs and WKY rats is due to increased Na^+/H^+ antiporter activity in SHRs. These current findings suggest that this increase in Na^+/H^+ antiporter activity is more likely

to be due to a change in the Km of the antiporter rather than to a change in the V_{max}, with a change in the Km of the antiporter in hypertension from pH 7.16 to 7.33.

In contrast to the changes found during muscle contraction, there was no evidence for increased Na^+/H^+ antiporter activity in the skeletal muscle of SHRs at rest.

No differences were found between adult SHRs and WKY rats in regard to intrinsic buffering, resting intracellular and extracellular pH, and resting intracellular and extracellular bicarbonate concentrations. In addition, there was also no evidence of a difference in skeletal muscle bicarbonate/chloride exchange between SHRs and WKY rats or resting lactate concentration.

The lack of difference in resting intracellular pH between the two types of rat, suggests that at rest differences in Na^+/H^+ antiporter activity due to a shift in Km of the antiporter are too small to result in a difference in resting pH.

Chapter seven

The effect of isoprenaline and nifedipine on *in vivo* Na^+/H^+ antiporter activity in rat skeletal muscle

7.1 INTRODUCTION

In Chapters 5 and 6 it was shown that activity of the Na^+/H^+ antiporter is increased in the skeletal muscle of the spontaneously hypertensive rat *in vivo*. It was also shown that the Na^+/H^+ antiporter is the most important mechanism responsible for regulating cytosolic pH during contraction-induced acidosis in skeletal muscle *in vivo* and this supports the work of others *in vitro* (Aickin and Thomas, 1977a; Juel, 1988). The work presented in this chapter is an extension of the work presented in Chapters 5 and 6 and concerns the investigation of the mechanism responsible for altering Na^+/H^+ antiporter activity in skeletal muscle.

Catecholamine release accompanying extracellular acidosis attenuates the reduction in cytosolic pH in both skeletal and cardiac muscle (Clancy et al., 1976). Furthermore, β-adrenoceptor agonists enhance the calcium current mediated by L-type calcium channels in skeletal muscle (Arreola et al., 1987). In view of these findings and the important role Na^+/H^+ exchange has in controlling acidosis in skeletal muscle, the hypothesis was proposed that one action of catecholamines on skeletal muscle was to increase skeletal muscle Na^+/H^+ antiporter activity. In addition, it was also felt possible that opening of L-type calcium channels in skeletal muscle could be linked to Na^+/H^+ antiporter activation in skeletal muscle.

In this work ^{31}Phosphorus nuclear magnetic resonance spectroscopy (^{31}P NMR) was again used to study Na^+/H^+ antiporter activity in the plantaris/gastrocnemius/soleus group of muscles in a rat leg during 10 Hz isometric contraction as described in chapters 2,5 and 6. In addition, the

effect of the β_2-adrenoceptor agonist isoprenaline and the effect of a specific L-type calcium channel blocker, the dihydropyridine calcium antagonist nifedipine, on *in-vivo* Na^+/H^+ antiporter activity in rat skeletal muscle was studied.

7.2 METHODS

7.21 Animals

13-week old male Wistar-Kyoto (WKY) rats ranging in weight from 200 g to 290 g, obtained from Olac Ltd, Bicester, UK were studied. Anaesthesia was induced with pentobarbitone sodium (30 mg/kg) and was maintained during each experiment with halothane (0.5-1.0 % in 1:1 N_2O:O_2) delivered through a face-mask. Sciatic nerve stimulation was used to cause contraction of the plantaris, gastrocnemius, and soleus muscles as a group.The method of preparing the rats for stimulation of the plantari/gastrocnemius/soleus muscle complex has been described previously (see Chapter 2).

Isoprenaline (0.1 mmol/kg) was used as a β_2-adrenoceptor agonist, nifedipine (0.8 mg/kg) as an L-type calcium channel antagonist, and amiloride (0.4 mmol/kg) as an inhibitor of the Na^+/H^+ antiporter. The drugs were given intra-arterially 30 min before muscle contraction.

The mean arterial pressure fell to 60 (SD 5) mmHg after all drug treatments. In order to examine whether or not this hypotension was responsible for the observed changes in cytosolic acid responses we

lowered the BP to 60 (SD 7) mmHg in a further group of animals by removing appproximately 2.5 ml of blood from an indwelling arterial catheter.

7.22 NMR spectroscopy

(see Chapter 2)

7.23 Measurements of metabolite concentrations

(see Chapter 2)

7.24 Statistical and mathematical methods

Tests of normality were performed on all data (Royston, 1983) and one- way ANOVA was used to analyse the data, using Dunnett's method to compare data obtained for the different drug treatments with un-treated rats and Fisher's Protected Least Significant Difference test to compare the different drug treatments. Areas under curves (AUCs) were calculated using Simpson's method.

7.3 RESULTS

7.31 The effects of isoprenaline, nifedipine, and amiloride on changes in cytosolic acid concentration during muscle contraction

Fig. 7.1a shows the cytosolic acid response to isometric contraction in untreated rats and in rats given amiloride (0.4 mmol/kg). In untreated rats the cytosolic acid concentration rose to a maximum after 120 s of stimulation and then returned to baseline, despite continued stimulation. Inhibition of the Na^+/H^+ antiporter by amiloride prevented this return to baseline.

Figs. 7.1b and 7.1c show the cytosolic acid responses to isoprenaline (0.1 mmol/kg) and nifedipine (0.8 mg/kg). Isoprenaline caused a significant reduction in both the peak cytosolic acid concentration achieved ($P < 0.001$) and the AUC between 0 and 600 s during isometric contraction ($P < 0.001$) compared with the untreated rats. In contrast, nifedipine did not significantly change either the maximum cytosolic acid concentration or the AUC between 0 and 600 s compared to untreated rats. However, the return to baseline acid concentrations was slower and the baseline acid concentration achieved during contraction was significantly greater than in untreated rats ($P < 0.001$).

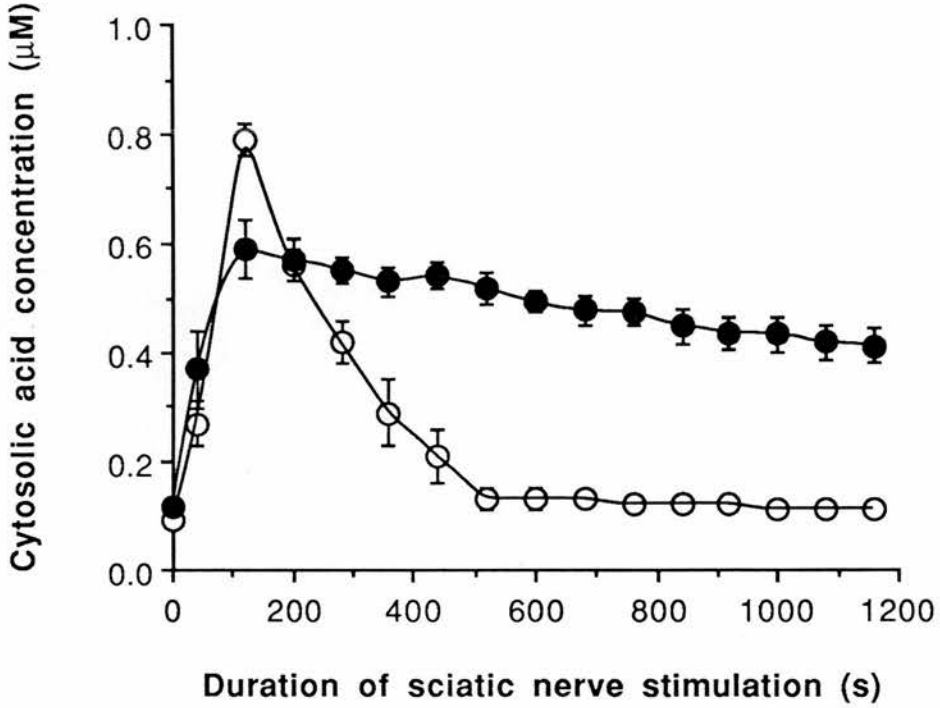
Both isoprenaline and nifedipine produced the same fall in arterial BP to 60 mmHg. Animals bled to the same mean BP produced a significant reduction in the maximum cytosolic acid concentration achieved

($P < 0.001$) compared with untreated rats, although comparison of the AUC between 0 and 600 s did not reach significance (Fig. 7.1d). Bleeding did not affect the rate of return to baseline acid concentrations and the baseline acid concentrations achieved were not significantly different from untreated rats.

Fig. 7.1e shows the cytosolic acid response after administration of the combination of isoprenaline (0.1 mmol/kg) and nifedipine (0.8 mg/kg) before contraction. This combination caused a significant reduction in the maximum cytosolic acid concentration achieved ($P < 0.001$) and a significant reduction in the AUCs between 0 and 600 s compared with untreated rats ($P < 0.004$) but not compared with rats given only isoprenaline. However, after the combination of nifedipine and isoprenaline the rate of return to baseline acid concentrations was again slower and the baseline acid concentrations achieved were greater than after isoprenaline on its own ($P < 0.001$).

Fig. 7.1f shows the effect of nifedipine in isolation and in combination with isoprenaline. In both cases there was a significant reduction in the rate of return of cytosolic acid concentrations to baseline.

(a)


Cytosolic acid concentration (μM)
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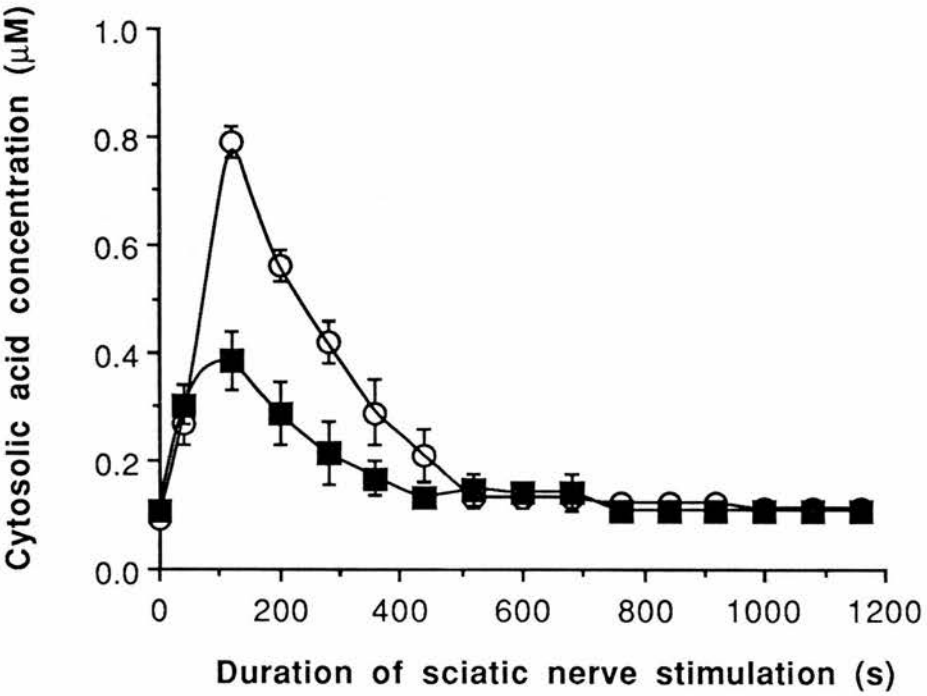
Duration of sciatic nerve stimulation (s)

(b)


Cytosolic acid concentration (μM)
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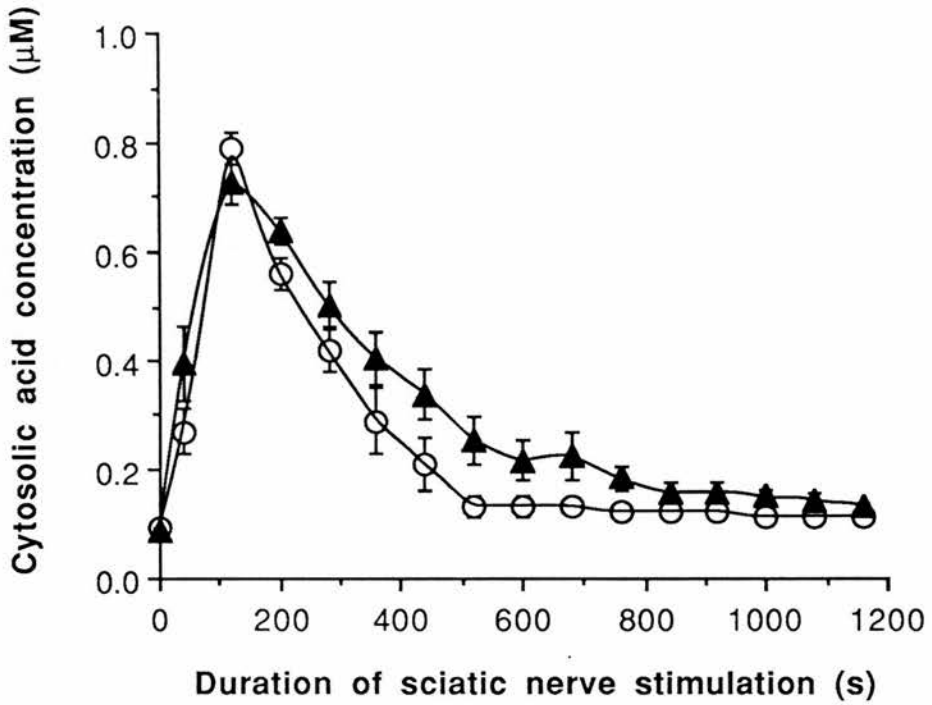
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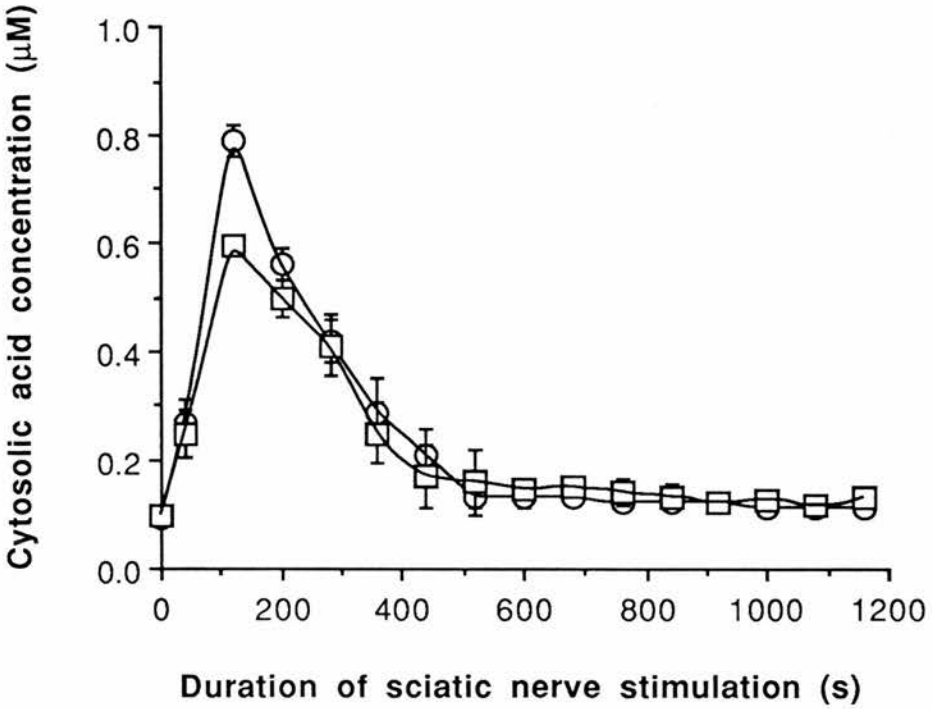
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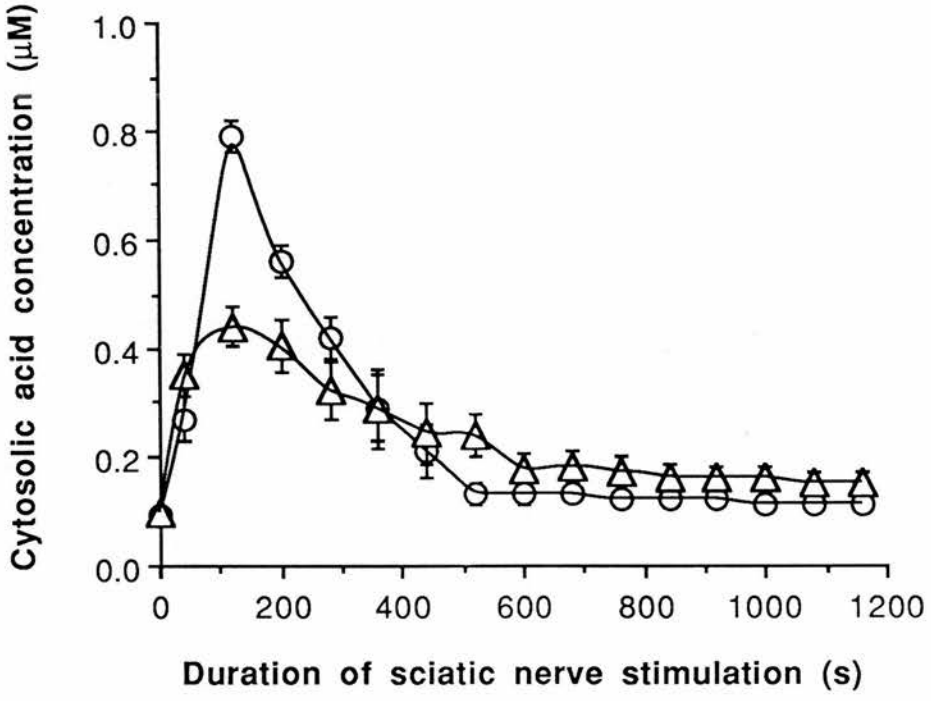
Duration of sciatic nerve stimulation (s)

(e)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Duration of sciatic nerve stimulation (s)

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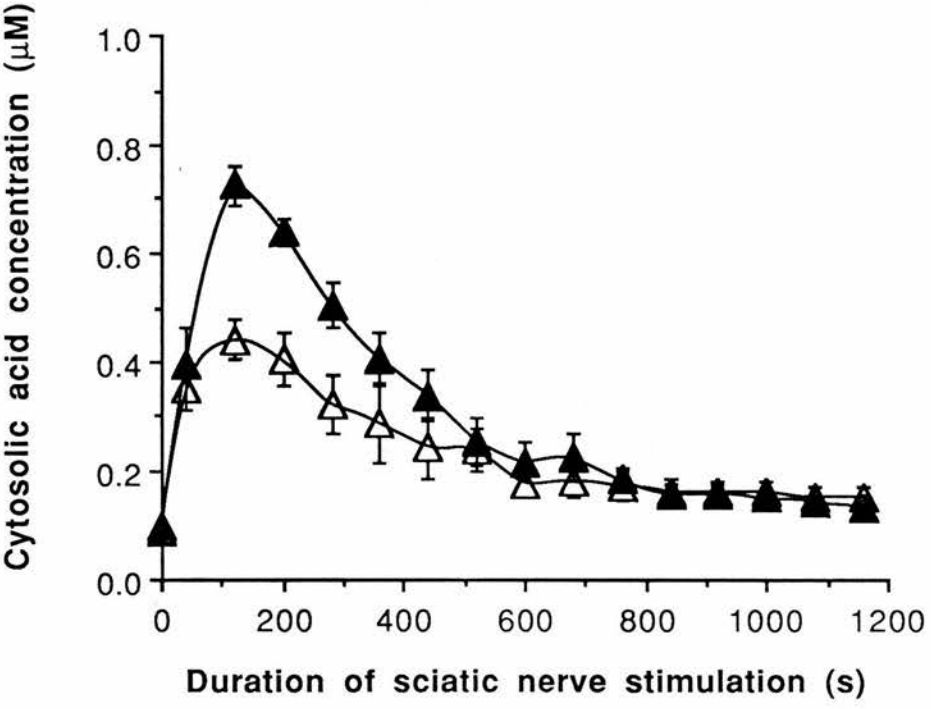
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Duration of sciatic nerve stimulation (s)

Fig. 7.1a-f. Changes in skeletal muscle cytosolic acid concentration (μM) (Mean ± SEM; n=6) during 10Hz isometric contraction in: (a) (○) untreated rats and (●) rats after amiloride (0.4mmol/kg) administration, (b) (○) untreated rats and (■) rats after isoprenaline (0.1 mmol/kg) administration, (c) (○) untreated rats and (▲) rats after nifedipine (0.8 mg/kg) administration, (d) (○) untreated rats and (□) rats after a 2.5 ml bleed, (e) (○) untreated rats and (△) rats after both isoprenaline (0.1 mmol/kg) and nifedipine (0.8 mg/kg) administration, (f) (▲) rats after nifedipine (0.8 mg/kg) administration and (△) rats after both 0.1 mmol/kg isoprenaline and 0.8 mg/kg nifedipine administration.

7.32 Changes in lactate, phosphocreatine, inorganic phosphate, and ATP during contraction

Table 7.1 shows the concentrations of muscle lactate at 0 and 120 s of contraction during sciatic nerve stimulation. Isoprenaline both alone and in combination with nifedipine resulted in a significant increase in resting muscle lactate concentrations compared with untreated rats ($P < 0.001$) and with rats given nifedipine alone ($P < 0.001$). Isoprenaline, nifedipine, and bleeding all resulted in significant increases in muscle lactate concentrations at 120 s of stimulation compared with untreated rats ($P < 0.001$). However, there was no significant difference in the increase in lactate concentrations in these different groups of animals. In contrast, treatment with amiloride (0.4 mmol/kg), which also caused a reduction of mean BP to 60 mmHg, did not result in an increase in muscle lactate concentrations compared to untreated rats.

Table 7.1. Muscle lactate (μmol/g dry weight) at rest and after 120 s isometric stimulation

	Muscle lactate	
Condition	at rest	after 120 s
untreated	13.2 (3.7)	63.7 (3.2)
after amiloride	12.8 (1.2)	57.6 (3.6)
after isoprenaline	18.5 (3.4)*	117.3 (7.6)†
after nifedipine	13.2 (2.8)	104.4 (7.5)†
after a 2.5 ml bleed	12.4 (1.5)	97.4 (5.3)†
after isoprenaline and nifedipine	20.5 (3.7)*	113.5 (15.1)†

Values are shown as mean (SD); (n=6)

*$P < 0\ 001$ compared with all unmarked values at 0 s (ANOVA with Dunnett's test). †$P < 0.001$ compared with all unmarked values at 120 s (ANOVA with Dunnett's test)

Fig. 7.2 shows that there was no significant difference in both the reduction and recovery of PCr during sciatic nerve stimulation in all groups of animals. There were also no significant differences in the change in P_i or the fall in ATP during contraction in the different groups of animals (data not shown). Both PCr and P_i act as important buffers in muscle cytosol during contraction (Wolfe et al., 1988).

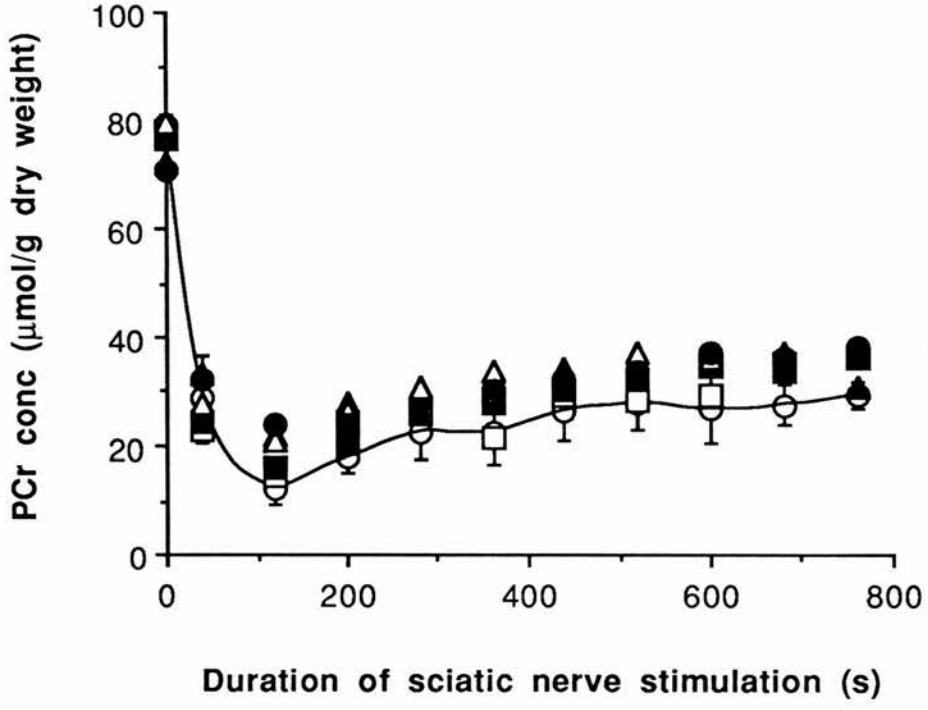


Fig 7.2 The change in skeletal muscle phosphocreatine (PCr) concentration (μmol/g dry weight) during 10 Hz isometric contraction in (○) untreated rats (Mean ± SD; n=6) and the mean values (n=6) of rats after administration of: (●) amiloride (0.4 mmol/kg), (■) isoprenaline (0.1 mmol/kg), (▲) nifedipine (0.8 mg/kg), (△) isoprenaline (0.1 mmol/kg) and nifedipine (0.8 mg/kg), and (□) after a 2.5 ml bleed.

7.33 Changes in twitch tension during contraction

Figs. 7.3a-f show the twitch tension response produced by the contracting skeletal muscle during 10 Hz isometric contraction. Stimulation at this frequency results in summation of contraction and partial tetanus, due to a build up of cytosolic calcium during contraction (Fig.7.3a). After amiloride relaxation between twitches was abolished (Fig. 7.3b).

Figs. 7.3c to 7.3f show that isoprenaline, nifedipine, and bleeding before contraction increased the relaxation between twitches and reduced the summation of contraction ($P < 0.001$).

7.4 DISCUSSION

As shown in chapters 5 and 6 the pattern of changes in cytosolic acid concentrations during skeletal muscle contraction can be used to study Na^+/H^+ antiporter activity, which is the most important mechanism for controlling acid response during contraction. This result is emphasized here by the effect of amiloride, an inhibitor of the Na^+/H^+ antiporter (Fig. 7.1a). In this work this technique has been used to study the effects of isoprenaline and nifedipine on *in vivo* Na^+/H^+ antiporter activity.

The effect of isoprenaline on Na^+/H^+ antiporter activity

Stimulation of β_2-adrenoceptors by isoprenaline reduced the maximum cytosolic acid concentration achieved during contraction (Fig. 7.1b), despite increasing skeletal muscle lactate concentrations (Table 7.1). This

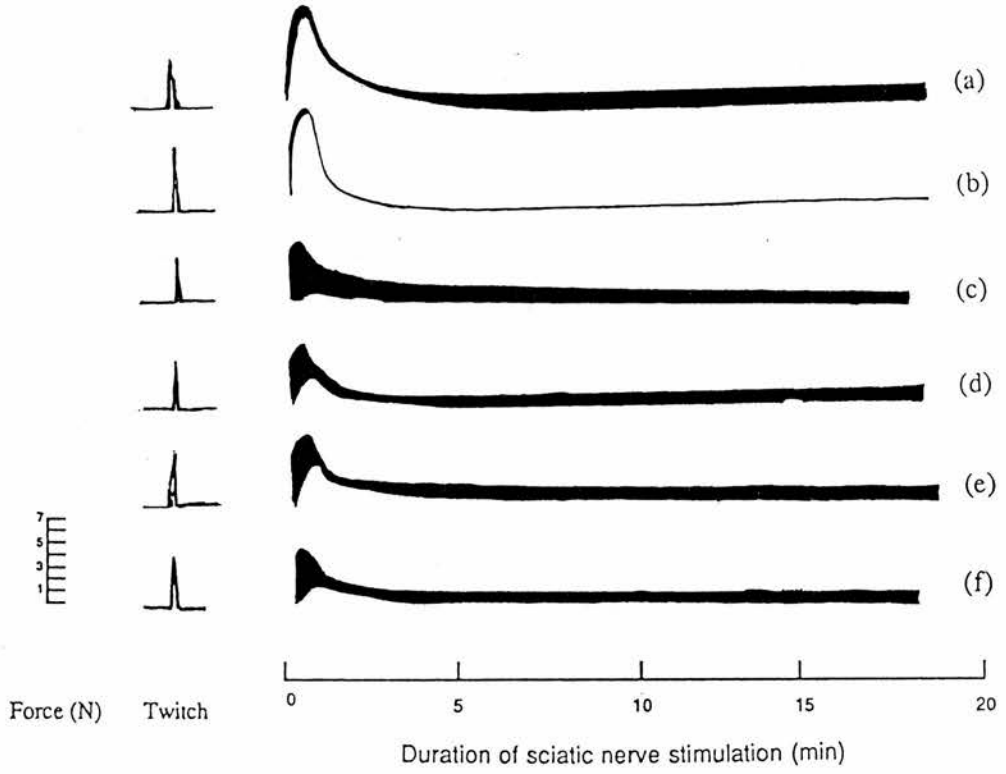


Fig. 7.3 Changes in the force of muscle contraction (N, newtons) during 10 Hz sciatic nerve stimulation in skeletal muscle of: (a) untreated rats, (b) rats after administration of amiloride (0.4 mmol/kg),(c) rats after administration of isoprenaline (0.1 mmol/kg), (d) rats after administration of nifedipine (0.8mg/kg), (e) rats after a 2.5 ml bleed, (f) rats after administration of both isoprenaline (0.1mmol/kg) and nifedipine (0.8mg/kg).

effect was not attributable to the lowering of blood pressure caused by isoprenaline, since the effect of isoprenaline was significantly greater than the effect produced by bleeding to the same BP (Fig. 7.1d) ($P < 0.001$). Thus, this finding is consistent with an increase in Na^+/H^+ antiporter activity in skeletal muscle in response to β_2-adrenoceptor stimulation. As β_2-adrenoceptor stimulation results in an increase in the concentrations of cyclic AMP in skeletal muscle, it is likely that a cyclic-AMP-dependent process is responsible for this increase in Na^+/H^+ antiporter activity. This is consistent with the finding that activation of a cyclic-AMP-dependent kinase increases Na^+/H^+ antiporter in frog erythrocytes (Borgese et al., 1987), although not with the finding that it inhibits the Na^+/H^+ antiporter in lymphocytes (Grinstein and Cohen, 1987).

7.42 The effect of nifedipine on Na^+/H^+ antiporter activity

In contrast to isoprenaline, nifedipine did not reduce the maximum cytosolic acid concentration, although it did reduce the rate of return to baseline during continued contraction. Furthermore, the fall in maximum cytosolic acid concentrations which occurred during contraction in animals who had been bled did not occur in the animals treated with nifedipine, despite the fact that the BP was the same in the two groups of animals.

The return of cytosolic acid concentrations to baseline during contraction was partially attenuated by nifedipine. Since this return to baseline, which is completely blocked by amiloride (Fig. 7.1a), is attributable to the

activity of the Na^+/H^+ antiporter (see Chapter 5 and 6) this result is consistent with a requirement of calcium for the full activation of the antiporter in skeletal muscle. This result is also consistent with the *in vitro* finding that in vascular smooth muscle calcium is required for full activation of the Na^+/H^+ antiporter (Mitsuhashi and Ives, 1988).

Furthermore, since the action of nifedipine as a calcium antagonist is specific to the L-type calcium channel (Flockerzi et al., 1986; McCall, 1987), this result suggests that one function of the L-channel in skeletal muscle is the provision of calcium for Na^+/H^+ antiporter activation.

7.43 The effect of bleeding on Na^+/H^+ antiporter activity

Hypotension secondary to bleeding caused a reduced maximum cytosolic acid response during contraction, despite an increased muscle lactate concentration. This suggests that hypotension induced by a bleed is also associated with an *increase* in Na^+/H^+ antiporter activity in skeletal muscle

Somatic nerves to skeletal muscle also contain sympathetic nerve fibres (Sundlöf et al., 1977). It has also been shown that hypotension due to bleeding results in increased sympathetic neuronal discharge in these nerves (Green and Heffron, 1966). It is likely therefore that the explanation for the decreased acid response during contraction seen in this study after a bleed results from an increase in Na^+/H^+ antiporter activity due to increased sympathetic discharge to skeletal muscle, resulting in increased β_2-adrenoceptor stimulation. This may also be one of the mechanisms responsible for the well-known but poorly understood "fright and flight"

response, since it is likely that increased sympathetic discharge to skeletal muscle prepares an animal for muscle contraction and flight by increasing Na^+/H^+ antiporter activity and H^+ ion efflux.

7.44 Differences in contractile responses after amiloride, isoprenaline, nifedipine, and bleeding

During contraction of skeletal muscle there are large increases in cytosolic calcium, and the two main mechanisms responsible for the bulk removal of calcium from the cytosol are the sarcolemmal Na^+/Ca^{++} exchanger and Ca^{++}-ATPase activity in the sarcoplasmic reticulum (Carafoli, 1987; Carafoli, 1988). Of the two, Ca^{++}-ATPase activity is thought to be the more important. At a sciatic nerve stimulation of 10 Hz used in this study there is summation of muscle contraction. This suggests that the mechanisms for controlling cytosolic calcium have been overcome, leading to accumulation of calcium during contraction and summation of contraction.

Amiloride produced almost complete inhibition of relaxation between twitches. The mechanism for this is uncertain but it is known that amiloride in high concentrations inhibits not only Na^+/H^+ antiporter activity but also Na^+/Ca^{++} exchange and Na^+/K^+-ATPase activity (for review see Frelin et al., 1987).Thus, inhibition of Na^+/Ca^{++} exchange and Na^+/K^+-ATPase activity may be the cause of the reduced relaxation after amiloride.

In contrast to amiloride, isoprenaline, nifedipine, and bleeding all caused a significant increase in relaxation between twitches and a reduced maximum generated tension in the muscle during contraction. The mechanism responsible for this increased relaxation is also uncertain but it is possible that this could be a cyclic-AMP-dependent process, as isoprenaline, nifedipine, and bleeding would all be expected to increase concentrations of cyclic-AMP. However, this is unlikely to involve a cyclic-AMP-dependent increase in sarcoplasmic Ca^{++}-ATPase activity as shown in myocardium (Limas and Spier, 1980), because there is no evidence for such a mechanism in skeletal muscle (Stull, 1980). Nevertheless, an increased cyclic-AMP concentration could increase Na^{+}/K^{+}-ATPase activity in skeletal muscle (Clausen, 1986) and increase relaxation by increasing the rate of repolarization following contraction.

There was no relationship between increased muscle relaxation during contraction and changes in cytosolic pH in the pH range 6.10 - 7.04. This suggests that in skeletal muscle changes in pH in this range have little effect on the action of calmodulin in regulating Ca^{++}-ATPase activity (Carafoli, 1987).

7.45 Changes in skeletal muscle metabolism after amiloride, nifedipine, isoprenaline, and bleeding

Amiloride had no effect on skeletal muscle concentrations of lactate during contraction, in contrast to nifedipine, isoprenaline, and bleeding, all of which increased muscle lactate concentrations during contraction (Table 7.1).

The difference between the effects on skeletal muscle lactate of amiloride on the one hand and of nifedipine, isoprenaline, and bleeding on the other hand may be due to inhibition of adenylate cyclase by amiloride (Mahé et al., 1985), since the other stimuli would all be expected to stimulate adenylate cyclase and increase cyclic-AMP concentrations. These results also show that differences in lactate production could not explain the differences in acid responses to nifedipine, isoprenaline, and bleeding (Figs 7.1a-f).

None of the stimuli used caused any changes in the utilization of skeletal muscle PCr (Fig. 7.2) or changes in P_i during contraction. This suggests that the differences in acid responses during contraction are not due to major differences in intracellular buffering from changes in P_i and PCr.

7.45 CONCLUSIONS

This work has shown that β_2-adrenoceptor stimulation increases the activity of the Na^+/H^+ antiporter *in vivo* in skeletal muscle. It has also shown that opening of the L-type calcium channel is necessary for full activation of the Na^+/H^+ antiporter in skeletal muscle *in vivo*. Furthermore, relaxation between twitches in skeletal muscle is increased in conditions that would be expected to increase cyclic-AMP and over the pH range 6.10 - 7.04 is not greatly affected by changes in pH.

Bleeding also causes an increase in the activity of the Na^+/H^+ antiporter *in vivo* in skeletal muscle. This is most likely to be due to an increase in

sympathetic nerve outflow to skeletal muscle as a result of hypotension. Thus, as part of the "fright and flight" reaction, this may be one of the mechanisms by which animals under stress prepare their skeletal muscle for exercise.

This work also has implications for the mechanism responsible for the finding of increased Na^+/H^+ antiporter activity in the skeletal muscle of SHRs (see Chapters 5 and 6). It is most likely that the increased Na^+/H^+ antiporter activity in skeletal muscle of SHRs is due to increased sympathetic nervous activity and increased β_2-adrenoceptor stimulation.

Chapter eight

The effect of nifedipine on *in-vivo* Na^+/H^+ antiporter activity in the spontaneously hypertensive rat

8.1 INTRODUCTION

Increased Na^+/H^+ antiporter activity has been found in blood cells and in vascular smooth muscle cells obtained from SHRs (Feig et al., 1987; Feig et al., 1986; Kuriyama and Aviv, 1987) and in blood cells obtained from patients with essential hypertension (Canessa et al., 1988; Livne et al., 1987). The finding of increased Na^+/H^+ antiporter activity in hypertension has led to the hypothesis that this increase may be responsible for the vascular smooth muscle hyperplasia and vascular remodelling which are important in the maintenance of increased blood pressure in patients with essential hypertension (Lever, 1986). The hypothesis that increased Na^+/H^+ antiporter is responsible for these changes in the vascular smooth muscle of SHRs and patients with essential hypertension stems from the finding that cell mitogens have been shown to increase Na^+/H^+ antiporter activity (Moolenar et al., 1982; Pouysségur et al., 1982). In addition, Na^+/H^+ antiporter activity has also been shown to increase with noradrenaline (Aalkjær and Cragoe, 1988) and angiotensin II (Berk et al., 1987a), which are known to cause vascular smooth muscle hyperplasia.

In Chapters 5-7 ^{31}Phosphorus nuclear magnetic resonance spectroscopy (^{31}P NMR) was used to make *in-vivo* measurements of cytosolic pH during 10 Hz isometric contraction of the plantaris/gastrocnemius/soleus group of muscles in a rat leg and it was found that the pattern of change in cytosolic pH during contraction is determined by Na^+/H^+ antiporter activity. This confirms the findings of others *in vitro*, that hydrogen ion efflux via the Na^+/H^+ antiporter is the most important

mechanism for controlling cytosolic pH in skeletal muscle (Aicken and Thomas, 1977). This method was used to assess Na^+/H^+ antiporter activity in the skeletal muscle of both SHRs and normotensive WKY rat controls and showed that Na^+/H^+ antiporter activity is increased in the skeletal muscle of SHRs *in vivo*, confirming *in-vitro* findings (see Chapters 5 and 6).

Work using cell mitogens suggests that there is a close association between increased cytosolic calcium and increased Na^+/H^+ antiporter activity (Busa and Nuccitelli, 1984), and in human fibroblasts stimulation of Na^+/H^+ antiporter by growth factors is blocked by calcium antagonists and calmodulin antagonists (Owen and Villereal, 1982). In hypertension, cells also demonstrate altered calcium handling and increased Na^+/H^+ antiporter (Postnov and Orlov, 1985). This has led to the hypothesis that an increase in cytosolic calcium concentration could in part be responsible for an increase in Na^+/H^+ antiporter activity in hypertension (Aviv, 1988). In support of this hypothesis it has been shown that in vascular smooth muscle cells full activation of the Na^+/H^+ antiporter by protein kinase C-dependent and independent pathways requires calcium (Mitsuhashi and Ives, 1988). In addition, there have been several reports that cytosolic calcium is raised in cells obtained from SHRs (Erne et al., 1984) and patients with essential hypertension (Astaire et al., 1989; Lindner et al., 1987). These same cells have been shown to have increased Na^+/H^+ antiporter activity (Feig et al., 1987; Feig et al., 1986; Kuriyama and Aviv, 1987; Canessa et al., 1988; Livne et al., 1987; Lever, 1986).

However, raised cytosolic calcium is not a uniform finding in association with hypertension (Nabika et al., 1985; Bukoski, 1990; Pritchard et al., 1989). Furthermore, the small increase in cytosolic calcium that has been reported in cells obtained from both SHRs and patients with hypertension would not necessarily explain the increased Na^+/H^+ antiporter activity found in vascular smooth muscle, as a much larger increase in cytosolic calcium would appear to be required for alteration of Na^+/H^+ antiporter activity in vascular smooth muscle cells (Mitsuhashi and Ives, 1988). Thus, even if cytosolic calcium is increased in cells obtained from hypertensive animals and humans this may still not explain the increased Na^+/H^+ antiporter activity found in association with hypertension.

However, cells obtained from hypertensive animals and humans which demonstrate increased Na^+/H^+ antiporter activity have shown a consistent increase in calcium influx via L-type calcium channels (Postnov and Orlov, 1985; Rusch and Hermsmeyer, 1988). It was considered therefore whether this increased calcium influx via potential operated calcium channels could, by leading to a *local* increase in calcium concentration at the plasma membrane (Carafoli, 1987), result in increased membrane Na^+/H^+ exchange in hypertension. To test this hypothesis ^{31}P NMR was used to measure *in-vivo* Na^+/H^+ antiporter activity and nifedipine, a specific L-type calcium channel antagonist, was used to determine the effect of blockade of L-type calcium channels on *in-vivo* Na^+/H^+ antiporter activity in SHRs.

8.2 METHODS

8.21 Animals

13-week old male SHRs and age-matched male WKY in-bred controls, ranging in weight from 240 g to 312 g, were obtained from Olac Ltd, Bicester, UK. Anaesthesia was induced with pentobarbital sodium (30 mg/kg) and was maintained during each experiment with halothane (0.5-1.0 % in 1:1 N_2O:O_2) delivered through a face-mask.

The method of preparing the rats for stimulation of the plantaris/gastrocnemius/soleus muscle complex has already been described (see Chapter 2). In experiments with nifedipine (Bayer U.K. Ltd) the drug was injected intra-arterially in a dose of 0.8 mg/kg 30-40 min before sciatic nerve stimulation.

Mean arterial pressure was monitored by an indwelling catheter in the external carotid artery and was 120 (SD 9) mmHg in the WKY rats (n = 20) and 185 (SD 9) mmHg in the SHRs (n = 20). The mean arterial pressure fell to 80 mmHg (SD 5; n = 6) in SHRs and 60 mmHg (SD 5; n = 6) after nifedipine. In order to determine whether or not hypotension was responsible for the observed changes in cytosolic acid responses the BP was lowered to 80 mmHg (SD 5; n=6) and 60 (SD 7; n=6) mmHg in further groups of SHRs and WKY rats respectively by removing approximately 2.5 ml of blood via an indwelling arterial catheter.

8.22 NMR spectroscopy

(see Chapter 2)

8.23 Measurements of metabolite concentrations

(see Chapter 2)

8.24 Statistical and mathematical methods.

The data are presented as means (SDs). Tests of normality were performed on all data (Royston, 1983) and one-way ANOVA was used to compare all groups of rats, using Fisher's Protected Least Significant Difference test. Areas under curves (AUCs) were calculated using Simpson's method.

8.3 RESULTS

8.31 The effects of nifedipine and bleeding on changes in cytosolic acid concentrations during muscle contraction.

Fig. 8.1a shows the cytosolic acid response to isometric contraction in untreated WKY rats and SHRs. In untreated rats the cytosolic acid concentration rose to a maximum after 120 s of stimulation and then returned to baseline concentrations, despite continued stimulation. The AUC between 0 and 600 s was significantly lower in the SHRs than in the WKY rats ($P = 0.006$). It has previously been shown that this dif-

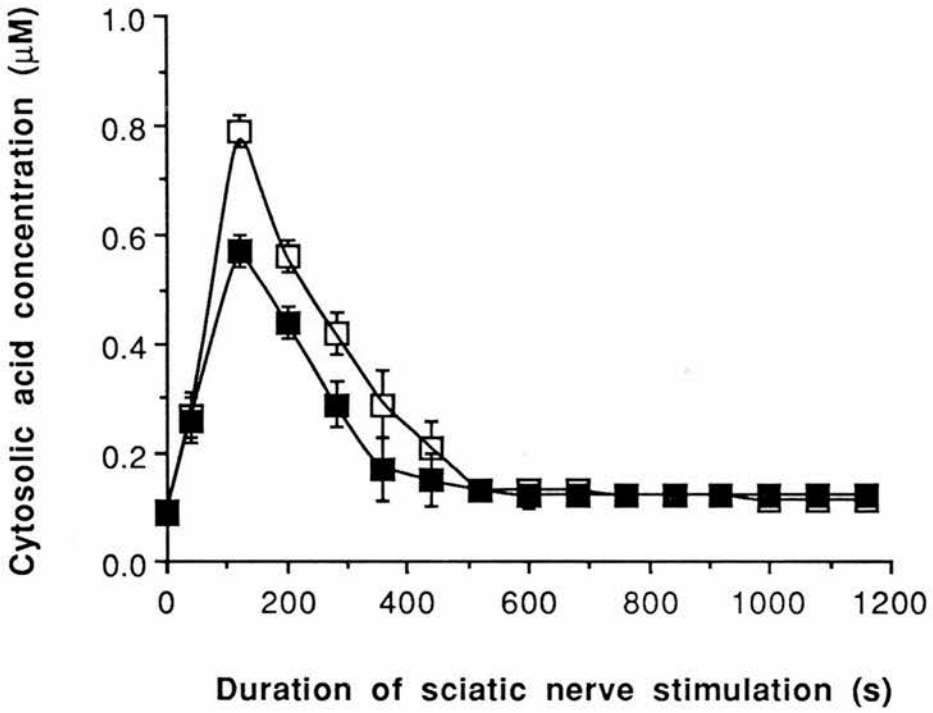
ference is removed by amiloride, an inhibitor of the Na^+/H^+ antiporter (see Chapter 5), which also prevented the return to baseline cytosolic acid concentrations.

Fig. 8.1b shows the cytosolic acid response in SHRs and WKY rats after nifedipine (0.8 mg/kg). Nifedipine removed the difference in AUC between untreated SHRs and untreated WKY rats. Nifedipine did not prevent the return to baseline concentrations of cytosolic acid but the rate of return to baseline acid concentrations was slower and the baseline acid concentration achieved during continued contraction was significantly higher than in untreated SHRs and WKY rats ($P < 0.001$).

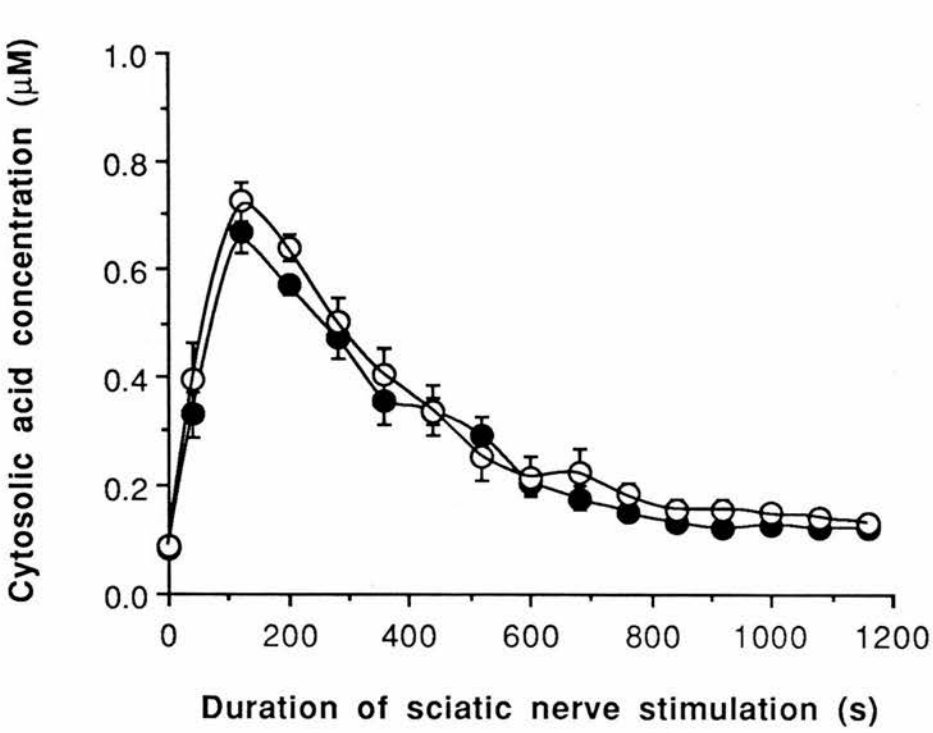
The BPs of the animals before treatment were 120 (9) mmHg in the WKY rats (n = 20) and 185 (9) mmHg in the SHRs (n = 20). Nifedipine produced a fall in mean arterial BP to 80 mmHg in the SHRs and to 60 mmHg in the WKY rats. The removal of 2.5 ml of blood resulted in similar falls in BP. However, in contrast to the effects of nifedipine (Fig. 8.1b), lowering the BP in this way did not remove the difference between the SHRs and WKY rats in the pattern of the cytosolic acid concentration changes during contraction, which remained significant ($P< 0.003$) (Fig. 8.1c).

When rats were bled before contraction the maximum cytosolic acid achieved in both SHRs and WKY rats was reduced ($P < 0.001$). However, in contrast to rats given nifedipine before contraction, reducing the blood pressure by bleeding did not affect either the rate of

(a)


Cytosolic acid concentration (μM)
1.0
0.8
0.6
0.4
0.2
0.0
0
200
400
600
800
1000
1200
Duration of sciatic nerve stimulation (s)

(b)


Cytosolic acid concentration (μM)
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Duration of sciatic nerve stimulation (s)

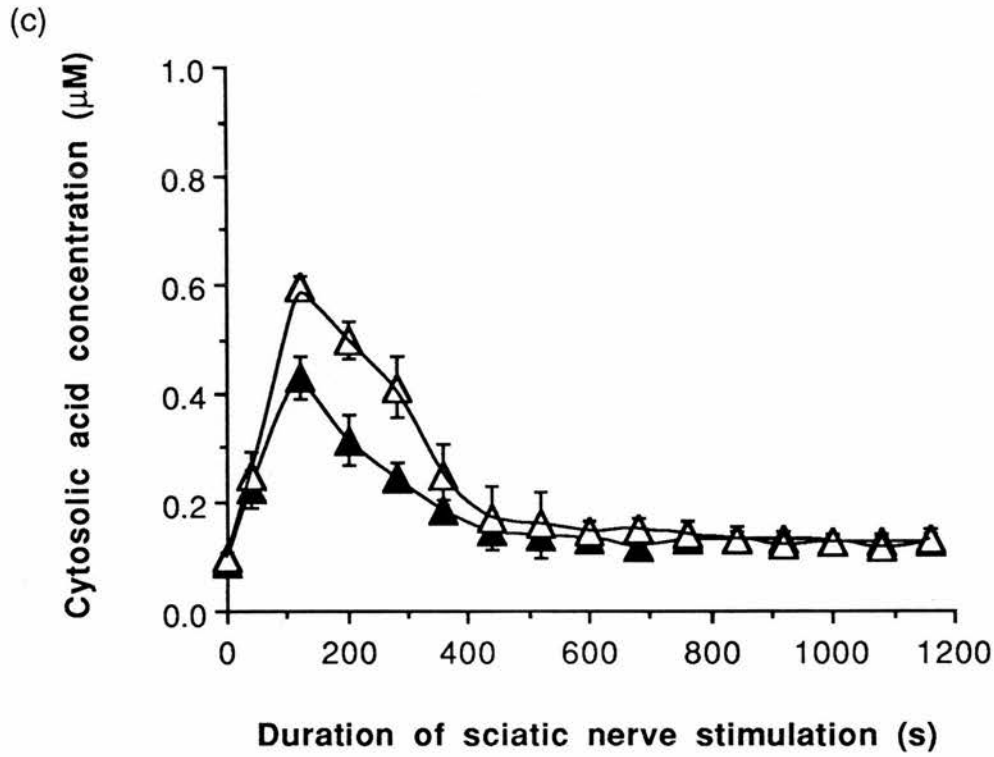

Fig. 8.1 The change in skeletal muscle cytosolic acid concentration (μM) (Mean with SEM; n=6) during 10 Hz isometric contraction in (a) untreated WKY rats (□) and untreated SHRs (■)(Fig. 5.1a), (b) WKY rats (○) and SHRs (●) after nifedipine (0.8 mg/kg), and (c) WKY rats (△) and SHRs (▲) after a 2.5 ml bleed.

return to baseline acid concentrations or the baseline acid concentration achieved during continued contraction.

8.32 Changes in lactate, phosphocreatine, inorganic phosphate, and ATP concentrations during contraction

Table 8.1 shows the concentrations of muscle lactate both at rest and after 120 s of contraction during sciatic nerve stimulation under

Table 8.1 Muscle lactate (μmol/g dry weight) at rest and after 120 s isometric contraction

	Muscle lactate					
Group	Untreated		After nifedipine		After bleeding 2.5 ml	
Rats	SHR	WKY	SHR	WKY	SHR	WKY
n	6	6	6	6	6	6
At rest	14.3 (3.9)	13.2 (3.7)	12.7 (1.7)	13.2 (2.8)	14.9 (1.5)	12.4 (1.5)
After 120 s	64.6 (14.5)	63.7 (7.9)	110.8 (15.9)*	104.4 (7.5)*	95.8 (6.0)*	97.4 (5.3)*

Values are shown as mean (SD); (n=6)

*$P < 0.001$ compared with untreated rats (ANOVA using Fisher's Protected Least Significant Difference test).

different experimental conditions. Both nifedipine and bleeding before contraction resulted in similar and significant increases in muscle lactate concentrations ($P < 0.001$). However, this increase in muscle lactate concentration was not significantly different between SHRs and WKY rats.

Fig. 8.2 shows that there was no significant difference in both the reduction and the recovery of PCr during sciatic nerve stimulation in all groups of animals. There was also no significant difference in the change in P_i or the fall in ATP concentrations during contraction in the different groups of animals (data not shown). Both PCr and P_i act as important buffers in muscle cytosol during contraction (Wolfe et al., 1988).

8.33 Changes in twitch tension during contraction.

Figs. 8.3a-f. show the typical twitch tension responses produced by the contracting skeletal muscle during 10 Hz isometric contraction in untreated WKY rats (Fig. 8.3a), untreated SHRs (Fig. 8.3b), WKY rats after nifedipine (Fig.8.3c), SHRs after nifedipine (Fig.8.3d), WKY rats after a 2.5 ml bleed (Fig. 8.3e), and SHRs after a 2.5 ml bleed (Fig. 8.3f). Stimulation at 10 Hz results in summation of contraction and partial tetanus, due to a build up of cytosolic calcium during contraction. The untreated SHRs showed increased relaxation between twitches and a reduced maximum generated tension compared with untreated WKY rats ($P < 0.001$), as has been previously shown (see Chapter 5). Both nifedipine and bleeding before contraction increased the degree of

relaxation between twitches and reduced the maximum tension generated by WKY rats ($P < 0.001$).

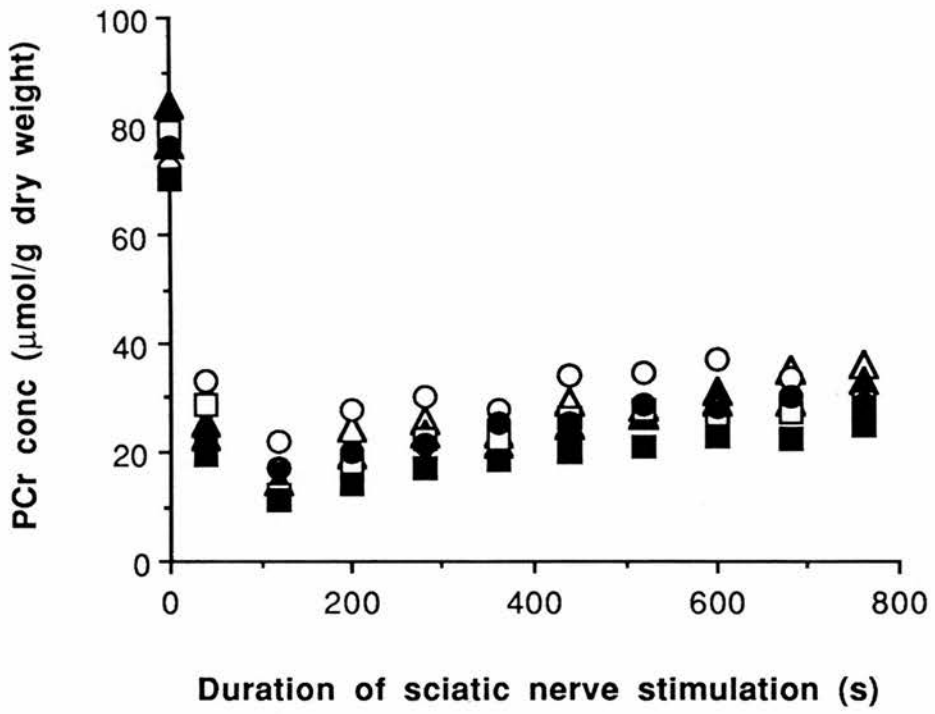

Fig. 8.2 The changes in skeletal muscle phosphocreatine (PCr) concentrations (μmol/g dry weight) during 10 Hz isometric contraction in (□) untreated WKY rats, (■) untreated SHRs, (○) WKY rats after nifedipine (0.8 mg/kg), (●) SHRs after nifedipine (△) WKY rats after a 2.5 ml bleed and (▲) SHRs after a 2.5 ml bleed. For clarity only the mean values are shown.

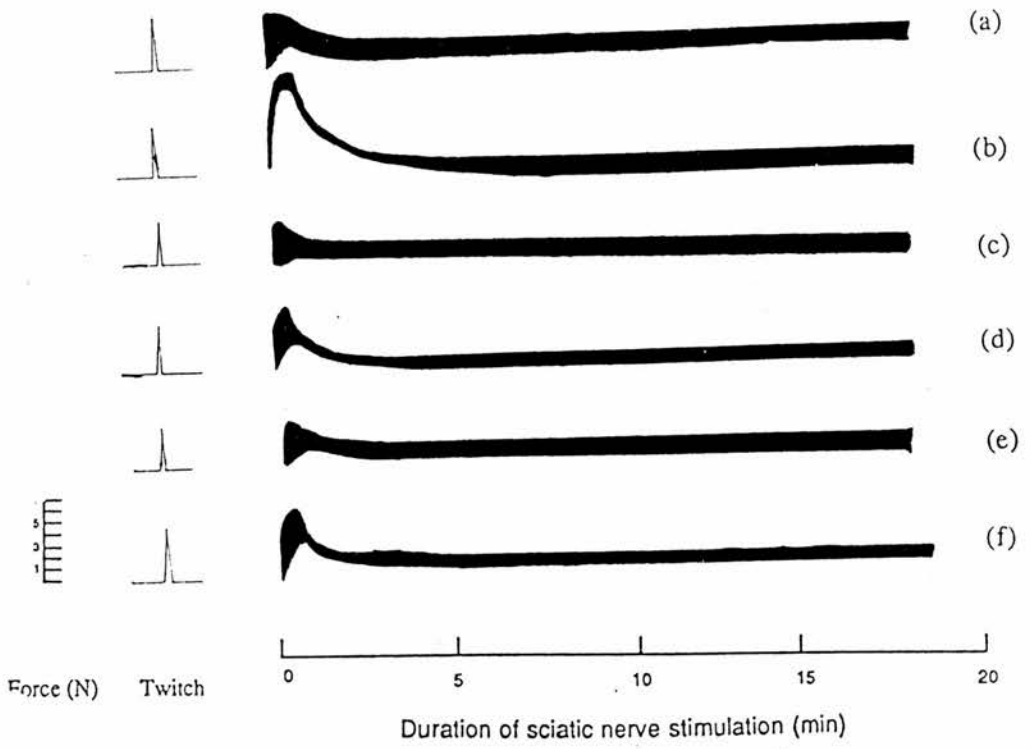

Fig. 8.3 Changes in the force of muscle contraction (N, newtons) during 10 Hz sciatic nerve stimulation in skeletal muscle of: (a) untreated SHRs, (b) untreated WKY rats, (c) SHRs after nifedipine (0.8mg/kg), (d) WKY rats after nifedipine (0.8mg/kg), (e) SHRs after a 2.5 ml bleed and (f) WKY rats after a 2.5 ml bleed.

8.4 DISCUSSION

As has been shown in previous chapters the pattern of changes in cytosolic acid concentrations during skeletal muscle contraction can be used to study Na^+/H^+ antiporter activity. (For the sake of clarity some of the data presented in Chapter 5 has been reproduced ie. Fig. 8.1a) In Chapter 5 it was shown that there is a significant difference in the cytosolic acid response in skeletal muscle in SHRs compared with WKY rats during 10 Hz isometric contraction, and that this difference is attributable to an increase in the activity of the Na^+/H^+ antiporter. In this study this technique has been used to delineate the role of L-type calcium channels in *in-vivo* Na^+/H^+ antiporter activity in the SHR, using nifedipine as a specific antagonist at those channels.

8.41 The effect of nifedipine on *in-vivo* Na^+/H^+ antiporter activity

After nifedipine the difference between the cytosolic acid responses in SHRs and WKY rats during contraction were no longer seen (Fig. 8.1b). This effect of nifedipine was not due to the fact that it caused hypotension, since the difference in the patterns of cytosolic acid responses between SHRs and WKY rats was not removed in animals that had been bled to the same BP (Fig. 8.1c).

Although nifedipine did not prevent the eventual return of cytosolic acid to baseline concentrations it did slow the *rate* of return. As the return to baseline cytosolic acid concentrations during continued contrac-

tion is determined by Na^+/H^+ antiporter activity (see Chapter 5), this result suggests that increased calcium influx via the opening of L-type calcium channels is required for full activation of the Na^+/H^+ antiporter *in vivo*. Furthermore, blockade of the L-type channel removed the difference in the cytosolic acid response between SHRs and WKY rats, and this suggests that the finding of increased Na^+/H^+ antiporter activity (see Chapters 5 and 6) in the skeletal muscle of the SHR is secondary to increased calcium influx via L-type calcium channels. In addition, as increased calcium influx via L-type calcium channels has been demonstrated in vascular smooth muscle cells obtained from SHRs compared with WKY rats (Rusch and Hermsmeyer, 1988), it is possible that increased calcium influx could also be the mechanism responsible for the increase in Na^+/H^+ antiporter activity found in vascular smooth muscle in hypertension.

If the increase in Na^+/H^+ antiporter activity in skeletal muscle in SHRs is due to increased calcium influx it could result from a *local* increase in the concentration of calcium at the cell membrane rather than a generalized increase in cytosolic calcium concentration (Carafoli, 1987). Indeed, it is unlikely that even a moderate increase in calcium influx into the muscle cell would result in a generalized increase in the cytosolic calcium concentration, because the amount of calcium entering the muscle cell via the L-type calcium channels is small (of the order of nmol) and the capacity of the muscle cell to remove calcium from the cytosol into sarcoplasmic reticulum is very large (of the order of mmol) (Carafoli, 1987; Carafoli, 1988).

8.42 The effect of reducing blood pressure by bleeding on *in-vivo* Na^+/H^+ antiporter activity

Hypotension secondary to bleeding caused a reduced maximum cytosolic acid response during contraction in both groups of animals, despite an increased muscle lactate concentration. This suggests that hypotension induced by a bleed is also associated with an increase in Na^+/H^+ antiporter activity in skeletal muscle. This was discussed in Chapter 7. This increase in Na^+/H^+ antiporter activity could be due to increased sympathetic nerve activity affecting skeletal muscle during bleeding. Somatic nerves to skeletal muscle contain sympathetic nerve fibres (Sündlof and Gunnar Wallin, 1977), and it has been shown that hypotension due to bleeding results in increased sympathetic neuronal discharge in these nerves (Green and Heffron, 1966). As there is also evidence of increased sympathetic neuronal activity in the SHR (Steikel et al., 1986), increased Na^+/H^+ antiporter activity in the skeletal muscle of SHRs most likely results from increased sympathetic neuronal activity in skeletal muscle and in conjunction with the work presented in Chapter 7 most likely occurs via increased β_2-adrenoceptor stimulation.

8.43 Changes in skeletal muscle metabolism after nifedipine and bleeding.

The differences in acid responses during contraction are not due to major differences in intracellular buffering from changes in PCr and P_i, since none of the stimuli used caused any changes in the utilization

of skeletal muscle PCr or a difference in the change in P_i concentration during contraction (Fig. 8.2). Although nifedipine and bleeding increased muscle lactate concentrations during contraction, differences in lactate production do not explain the differences in cytosolic acid responses to nifedipine and bleeding (Figs. 8.1b and 8.1c).

8.44 Differences in contractile responses between untreated SHRs and WKY rats and the effects of nifedipine and bleeding.

Data presented in Chapter 5 has been reproduced here for the sake of clarity (Figs. 8.3a and b), which shows that there is increased relaxation after contraction in skeletal muscle in SHRs. This has also been shown in subcutaneous arterioles obtained from patients with essential hypertension (Aalkjær et al., 1989). This increased relaxation could be due to increased bulk calcium extrusion from either increased Ca^{++}-ATPase activity in the sarcoplasmasic reticulum or increased Na^+/Ca^{++} exchange (Carafoli, 1987) (as discussed in Chapter 5). Alternatively, it could be due to an increased rate of repolarization secondary to increased activity of Na^+/K^+-ATPase, which would be consistent with the findings presented in Chapter 4 of increased *in vivo* Na^+/K^+-ATPase activity in the skeletal muscle of SHRs.

The difference in relaxation after contraction between SHRs and WKY rats was removed by nifedipine and bleeding (Figs. 3c-f). It is not possible to be certain as to the underlying mechanism of this effect, but increased relaxation may be secondary to the hypotension caused by

nifedipine and bleeding which would result in an increased sympathetic discharge to skeletal muscle. As increased sympathetic activity has been shown to increase Na^+/K^+-ATPase activity in skeletal muscle (Clausen, 1986) this may be the mechanism responsible for the increased rate of muscle relaxation seen after nifedipine and bleeding.

CONCLUSIONS

In summary, this work shows that there is increased Na^+/H^+ antiporter activity *in vivo* in the skeletal muscle of SHRs compared with WKY rats and that this difference in antiporter activity can be removed by blocking L-type calcium channels with nifedipine. This is evidence that the increased activity of the Na^+/H^+ antiporter found *in vivo* in the skeletal muscle of the SHR occurs in response to increased calcium influx via L-type calcium channels. As increased calcium influx and increased Na^+/H^+ antiporter activity have been shown to occur together in other cells in hypertension, including vascular smooth muscle, increased calcium influx via L-type calcium channels may also be the mechanism responsible for the finding of increased Na^+/H^+ antiporter activity in vascular smooth muscle. Furthermore, increased activation of Na^+/H^+ antiporter due to increased calcium influx could occur without any increase in cytosolic calcium concentrations (as discussed above).

Chapter nine

Summary and Conclusions

9.1 SUMMARY AND CONCLUSIONS

In this thesis I have described new ways of studying ion transport *in vivo* using nuclear magnetic resonance spectroscopy. These new techniques have been developed in order to study ion transport in hypertension and thus to determine whether or not *in vitro* abnormalities of ion transport are present *in vivo* in hypertension. The main findings from this work are listed in Table 9.1.

In Chapter 3 a new method of measuring *absolute* concentrations of the cation rubidium in intact animals using ^{87}Rb NMR (^{87}Rubidium nuclear magnetic resonance spectroscopy) was described. NMR to date, has been used as a qualitative tool and previous methods of quantification have made assumptions about tissue volume. The method described in chapter 3 makes no assumptions regarding tissue volume and overcomes problems of sample geometry, sample volume, tissue conductivity, coil tuning and amplifier gain. This method can be applied to other NMR-visible nuclei and so could have many biological applications and could be applied to man.

In Chapter 4 experiments were described which used the above method to measure changes in tissue rubidium concentration with time during the administration of rubidium salts, in the same animal. The aim of this study was to assess the *in vivo* activity of sodium- and potassium-activated adenosine triphosphatase (Na^+/K^+-ATPase) in the skeletal muscle of the spontaneously hypertensive rat.

These experiments showed that the rates of influx and efflux of rubidium are equally increased in skeletal muscle in SHRs *in vivo*. Furthermore, the increases in the rates of rubidium influx and efflux are balanced, so that there is no difference in the steady-state concentrations of rubidium in either skeletal muscle or erythrocytes. These findings suggest that in skeletal muscle and erythrocytes of SHRs there is increased Na^+/K^+-ATPase activity and an increased rate of K^+ efflux. Furthermore, SHRs had a faster redistribution of skeletal muscle rubidium than WKYrats, and this could be explained by increased Na^+/K^+-ATPase activity in tissues other than skeletal muscle and erythrocytes in the SHR.

These *in-vivo* findings of *increased* Na^+/K^+-ATPase activity are against the hypothesis that *reduced* Na^+/K^+-ATPase activity is responsible for increasing vascular tone in hypertension.

Na^+/H^+ antiporter activity *in vitro* has been found to be increased in cells, including blood cells and vascular smooth muscle cells obtained from both SHRs and patients with essential hypertension. In Chapters 5 and 6 experiments were described which showed that Na^+/H^+ antiporter activity is also increased in skeletal muscle of SHRs *in vivo*. The increase in Na^+/H^+ antiporter activity found in the SHR *in vivo* is more likely to be due to a change in the Km rather than to a change in the V_{max} of the antiporter, with a change in the Km of the antiporter in hypertension from pH 7.16 to 7.33.

It is also clear from this work that increased Na^+/H^+ antiporter activity is found in association with increased relaxation of skeletal muscle following contraction. This is consistent with the finding of increased relaxation of vascular smooth muscle following contraction found in arterioles obtained from patients with essential hypertension (Aalkjær et al., 1989). These findings are against the hypothesis that increased Na^+/H^+ antiporter activity is responsible for increasing vascular smooth muscle contractility in hypertension. Furthermore, in view of the increased vascular relaxation, the cause of increased/normal vascular tone in hypertension is likely to be due to increased activity of endogenous contractile agonists.

In Chapter 6 experiments were described which showed that the changes in cytosolic acid following contraction were unaffected by 20% inhaled carbon dioxide or by 4,4´-di-isothiocyanostilbene-2,2´-disulphonate (DIDS). This is evidence that bicarbonate exchange *in vivo* is not important in the control of cytosolic acid concentrations during skeletal muscle contraction in either SHRs or WKY rats.

Furthermore, no differences were found between adult SHRs and WKY rats in regard to intrinsic buffering, resting intracellular and extracellular pH, and resting intracellular and extracellular bicarbonate concentrations. In addition, there was no evidence of a difference in skeletal muscle bicarbonate/chloride exchange between SHRs and WKY rats.

These results show that SHRs and WKY rats have the same resting lactate production, intrinsic buffering, bicarbonate/chloride exchange, and arterial *Pa* CO_2. This explains the lack of difference in resting intracellular pH between the two types of rat and suggests that at rest differences in Na^+/H^+ antiporter activity due to a shift in Km of the antiporter are too small to result in a difference in resting pH.

In Chapter 7 experiments were described which showed that β_2-adrenoceptor stimulation increases the activity of the Na^+/H^+ antiporter *in vivo* in skeletal muscle. It was also shown that opening of the L-type calcium channel is necessary for full activation of the Na^+/H^+ antiporter in skeletal muscle *in vivo*. Furthermore, relaxation between twitches in skeletal muscle is increased in conditions that would be expected to increase cyclic-AMP and over the pH range 6.10 - 7.04 is not greatly affected by changes in pH.

Bleeding also causes an increase in the activity of the Na^+/H^+ antiporter *in vivo* in skeletal muscle. This is most likely to be due to an increase in sympathetic nerve outflow to skeletal muscle as a result of hypotension. Thus, as part of the "fright and flight" reaction, this may be one of the mechanisms by which animals under stress prepare their skeletal muscle for exercise.

It is also clear from this work that the finding of increased Na^+/H^+ antiporter activity in the skeletal muscle of the spontaneously hypertensive rat is most likely to be due to increased sympathetic nervous activity and increased β_2-adrenoceptor stimulation.

In Chapter 8 experiments were described which showed that the increase in the activity of the Na^+/H^+ antiporter activity *in vivo* in the skeletal muscle of SHRs compared with WKY rats can be removed by blocking L-type calcium channels with nifedipine. This is evidence that the increased activity of the Na^+/H^+ antiporter found *in vivo* in the skeletal muscle of SHRs occurs in response to increased calcium influx via L-type calcium channels. As increased calcium influx and increased Na^+/H^+ antiporter activity have been shown to occur together in other cells in hypertension, including vascular smooth muscle, increased calcium influx via L-type calcium channels may also be the mechanism responsible for the finding of increased Na^+/H^+ antiporter activity in vascular smooth muscle. Furthermore, increased activation of Na^+/H^+ exchange due to increased calcium influx could occur without any increase in cytosolic calcium concentrations because of the high affinity and capacity of sarcoplasmic-Ca^{++}-ATPase.

In conclusion (see Table 9.1), I have described a series of experiments which were designed to study ion transport *in vivo* in hypertension. I have shown that ion transport abnormalities occur *in vivo* in hypertension and have provided evidence that both Na^+/K^+-ATPase activity and Na^+/H^+ antiporter activity are increased *in vivo* in the SHR. I have also shown that β_2-adrenoceptor stimulation is the mechanism by which Na^+/H^+ antiporter activity is increased in skeletal muscle and that increased Na^+/H^+ antiporter activity *in vivo* in SHRs is due to increased calcium influx via L-type calcium channels. Furthermore, I have provided evidence which may explain why SHRs have the same steady-state concentrations of intracellular potassium ions,

hydrogen ions and bicarbonate despite evidence for increased potassium and hydrogen ion flux. I have also shown that like vascular smooth muscle, increased relaxation of skeletal muscle occurs following contraction and that increased relaxation following contraction is a feature of both vascular smooth muscle and skeletal muscle in hypertension. I have argued that this increased relaxation occurs as a result of increased Na^+/K^+-ATPase activity causing an increased rate of membrane repolarization and that if increased vascular tone does occur in hypertension it can only occur as a result of increased agonist-induced contraction.

In the final chapter of this thesis I will propose a new hypothesis that explains my findings of ion transport abnormalities in SHRs *in vivo* and the findings of others both *in vitro* and *in vivo* in blood cells and other cells obtained from patients with essential hypertension. This hypothesis will also attempt to explain the central role of the kidney in hypertension, the development of vascular hypertrophy, the development of increasing blood pressure with age and the finding of variable renin activity in association with essential hypertension.

Table 9.1 The main findings of the experiments presented in Chapters 4-8 regarding *in vivo* ion transport in the skeletal muscle of the spontaneously hypertensive rat compared with the Wistar-Kyoto rat.

An equal increase in both rubidium (potassium) influx and efflux with the same steady state rubidium (potassium) concentrations
Increased Na^+/K^+-ATPase activity
Increased Na^+/H^+ antiporter activity
Blocking L-type calcium channels removes the increase in Na^+/H^+ antiporter activity
No differences in resting pH, intrinsic buffering or bicarbonate handling

Chapter ten

A new hypothesis explaining ion transport abnormalities in essential hypertension

10.1 INTRODUCTION

Over the last few years there have been numerous reports of abnormal handling of cations by various cells obtained from patients with essential hypertension and from hypertensive rats. In this chapter I will present a hypothesis which attempts to explain the mechanism responsible for these abnormalities and the significance of these changes in cation handling to the development of genetic hypertension in rat and man. The hypothesis that will be presented incorporates other published hypotheses, namely the nephron hypothesis of Sealey et al. (1988) and the hypothesis of Lever (1986) that vascular smooth muscle hypertrophy is linked to an effect of angiotensin II. This hypothesis also explains other features of hypertension, including saline-induced natriuresis, variable plasma renin activities and differing salt sensitivities of patients with essential hypertension, and the tendency of blood pressure to increase with advancing age.

10.11 Abnormalities of ion transport *in vitro* in hypertension

10.111 *Intracellular ion concentrations*

There have been numerous reports of altered concentrations of intracellular cations in association with hypertension. Intracellular sodium concentrations have been reported to be consistently increased in leucocytes obtained from patients with essential hypertension (Edmondson et al., 1975; Ambrosioni et al., 1981; Heagerty et al., 1982). However,

in erythrocytes intracellular sodium concentrations have been reported to be either increased (Clegg et. al., 1982; Cole, 1983) or normal (Canessa et al., 1980; Garay et al., 1980) (for a review see Swales, 1982), although it has been argued that the intracellular sodium concentration as a mean of all studies is significantly increased (Hilton, 1986). However, there is growing evidence that much of what was thought to be intracellular sodium is extracellular, attached to the outside of the cell membrane, resulting in an apparent increase in intracellular sodium, and that intracellular sodium concentrations are probably normal in essential hypertension (Simon, 1989; Simon, 1990).

In contrast, intracellular potassium concentrations, which are much higher than intracellular sodium concentrations, and so less susceptible to measurement error, have not been found to be different in cells obtained from either patients with essential hypertension or spontaneously hypertensive rats (SHRs) (Boon et al., 1986; Aalkjær et al., 1985). This lack of difference in steady-state potassium concentrations occurs despite evidence that the rates of potassium fluxes into and out of cells are increased in genetic hypertension (Postnov et al., 1976; Orlov et al., 1989; see Chapter 4).

Small increases in free cytosolic calcium concentration have been reported in some cells (Erne et al., 1984; Astaire et al., 1989; Lindner et al., 1987), but this is not a uniform finding (Nabika et al., 1985; Bukoski, 1990; Pritchard et al., 1989). However, *total* cell calcium *has* been shown to be consistently increased in hypertension (Postnov and Orlov, 1985; Tobian and Chesley, 1966; Speiker et al., 1988). Total

cellular calcium increases with age (Bhalla et al., 1978; Fleckenstein et al., 1986) as it does in hypertension, but the process of calcium sequestration is accelerated in hypertension (Speiker et al., 1988; Bruschi et al., 1985).

10.112 *Abnormalities of ion transport systems*

Numerous abnormalities have been described in a variety of ion transport systems in hypertension, including Na^+/K^+-ATPase (the Na^+/K^+ pump), Na^+/Na^+ (Na^+/Li^+) exchange, the Na^+/H^+ antiporter, L-type calcium channels, sarcolemmal Ca^{++}-ATPase, and $Na^+/K^+/Cl^-$ co-transport.

Na^+/K^+ pump activity has been proposed to be reduced in leucocytes (Edmondson et al., 1975; Poston et al., 1981; Heagerty et al., 1982). However, measurements of Na^+/K^+ pump activity in these experiments have relied on measurements of increased intracellular sodium concentrations and measurements of fluxes derived there from, and as mentioned above there is now some doubt that the intracellular sodium concentration really is increased in essential hypertension. This casts doubt on the suggestion that Na^+/K^+ pump activity is reduced in hypertension. Furthermore, there is other *in vitro* evidence that Na^+/K^+ pump activity is *increased* (Simon et al., 1987) in essential hypertension and in leucocytes of borderline hypertensives (Nielson et al., 1988). Confirmatory evidence of increased Na^+/K^+pump activity comes from *in-vivo* studies (see next section). It should be noted that increased Na^+/K^+ pump activity in these circumstances would not

necessarily imply that the intracellular sodium concentration should be reduced, since there is also evidence of increased sodium influx via the Na^+/H^+ antiport (see below).

In contrast to the confusion about the nature of the changes in Na^+/K^+ pump activity in essential hypertension, the changes which have been described in other transport systems in a variety of tissues have mostly been consistent. For example, there is consistent *in vitro* and *ex vivo* evidence of increases in the activities of the Na^+/Na^+ (Na^+/Li^+) countertransport (Canessa et al., 1980; Canessa et al., 1988), the Na^+/H^+ antiport (Livne et al., 1987; Kuriyama and Aviv, 1987, Izzard and Heagerty, 1989; for review see Aalkjær, 1990), increased calcium influx via L-type calcium channels (Rusch and Hermsmeyer, 1988; for reviews see Postnov and Orlov, 1985 and Dominiczak and Bohr, 1990), and reduced sarcolemmal Ca^{++}-ATPase activity (for reviews see Postnov and Orlov, 1985 and Dominiczak and Bohr, 1990). Some of these abnormalities have been confirmed *in vivo* (see below). The co-transport of $Na^+/K^+/Cl^-$ has been shown to be reduced at least in some cells, but the findings are inconsistent (for a review see Chipperfield, 1986).

10.12 Abnormalities of ion transport *in vivo* in hypertension

There are many difficulties in the techniques which are used to define ion transport abnormalities *in vitro* (Poston et al., 1982; Duhm et al., 1982; Boon et al., 1986; Simon, 1990). It has therefore been proposed that inconsistencies in the reports of *in vitro* ion transport abnormalities in hypertension may result from differences in the experimental conditions used in different laboratories (Swales, 1982; Boon et al., 1986; Neilsen et al., 1988).

10.121 *Cellular studies*

Problems with *in vitro* methods of studying cation transport have prompted the development over the last few years of methods of studying cation transport *in vivo*. These methods have been based on the principle that certain ions which are not found in high concentrations in the body can be used as markers for endogenous ions, for example rubidium for potassium (Boon et al., 1986) and lithium for sodium (Brearley et al., 1988). In these experiments animals or man are given an amount of the relevent ion of interest and blood samples taken at various times after cation administration. The concentration of the cation in plasma and erythrocytes are then determined *in vitro*. This enables an indirect estimate of cation handling *in vivo*. In essential hypertension these techniques have shown that there is increased Na^+/K^+-ATPase activity in erythrocytes and an increase in the rate of potassium absorption (Boon et al., 1986). In addition, there is an in-

crease in the activity of the Na^+/Li^+ countertransport in erythrocytes, which is associated with a change in either the stoichiometry or co-operativity of the transporter (Brearley et al., 1988).

10.122 *Intact tissue studies*

The work presented in the preceding chapters of this thesis has used nuclear magnetic resonance spectroscopy in new ways to study ion transport non-invasively and entirely *in vivo* (see Chapters 2-8). Part of this work has also used the concept that an *exogenous* ion (rubidium) can act as a marker of the way an *endogenous* ion (potassium) is handled *in vivo*. The techniques described in earlier chapters of this thesis have been used to measure transmembrane fluxes of rubidium and hydrogen ions in the skeletal muscle of intact spontaneously hypertensive rats. This work has led me to formulate a new hypothesis to explain why there are abnormalities of ion transport systems in hypertension.

10.2 PREVIOUS HYPOTHESES RELATING ABNORMALITIES OF ION TRANSPORT TO THE PATHOPHYSIOLOGY OF HYPERTENSION

Numerous hypotheses have been proposed to explain some of the ion transport abnormalities found in hypertension.

10.21 The hypothesis of inhibition of Na^+/K^+-ATPase activity

DeWardener and Macgregor (de Wardener and MacGregor, 1980) adapted a hypothesis of Dahl and proposed that a circulating inhibitor of Na^+/K^+-ATPase was responsible for increasing vascular tone in hypertension via an alteration of Na^+/Ca^{++} exchange in vascular smooth muscle, based on a mechanism first outlined by Blaustein (Blaustein, 1977). Subsequently many groups have identified Na^+/K^+-ATPase inhibitory activity or digitalis-like immunoreactivity in essential hypertension (for reviews see de Wardener and Clarkson, 1985 and Poston, 1987). However, the finding of increased rather than decreased Na^+/K^+-ATPase activity mentioned above is incompatible with this hypothesis. Furthermore, it has been difficult to find a mechanism which links inhibition of Na^+/K^+-ATPase activity to the development of an increase in blood pressure (Aronson, 1984; Casteels et al., 1985; Postnov and Orlov, 1985).

10.22 The hypothesis that generalized membrane abnormality is responsible for ion transport abnormalities in hypertension

It has been shown that the membranes of different cells obtained from patients with essential hypertension are abnormal, with evidence of reduced membrane fluidity and calcium binding (for reviews see Swales, 1982 and Postnov and Orlov, 1985). The finding of an abnormal cell membrane in association with multiple abnormalities of membrane ion transport has led to a major alternative proposal to that of De Wardener & MacGregor (1980) that an intrinsic defect of the cell membrane is responsible for the abnormalities in ion transport found in association with hypertension.(Swales, 1982; Postnov and Orlov, 1985; Postnov, 1990; Dominiczak and Bohr, 1990). For example, this has been proposed to be the mechanism for the abnormalities in the Na^+/H^+ antiport which are found in hypertension (Canessa, 1988). However, I have presented work in this thesis which shows that the increase in Na^+/H^+ antiport activity found in SHRs *in vivo* can be removed by blocking L-type calcium channels (see Chapter 8), suggesting that the antiporter is altered in hypertension by an interaction with calcium and not as a result of an interaction with an abnormal membrane. In addition, as will be outlined later, the findings of increased calcium influx and reduced sarcolemmal Ca^{++}-ATPase activity in essential hypertension, both of which have been considered to be due to an intrinsic membrane abnormality (Postnov and Orlov, 1985; Postnov, 1990; Dominiczak and Bohr, 1990) can result from increased agonist stimulation of the cell. Futhermore, the minor changes in

membrane composition found in essential hypertension are unlikely to be responsible for the finding of increased Na^+/K^+pump activity, as major changes in membrane composition induced artificially are required before Na^+/K^+-ATPase activity is altered (Skou, 1975) and any change in membrane composition is likely to lead to *reduced* rather than *increased* Na^+/K^+ pump activity (for review see Swales, 1982). Finally, it is equally likely that the ion transport abnormalities found in association with hypertension *cause* the changes in the composition of cell membranes, because of alterations in transcriptional, translational and post-translational events in the cell (for a review see Geering, 1986).

10.23 The hypothesis that renal ischaemia affects sodium reabsorption

A hypothesis has been proposed (Sealey et al., 1988) which attempts to explain the paradox that there is on the one hand increased sodium reabsorption from the proximal tubule of the kidney in essential hypertension but on the other hand low or normal plasma volume and blood volume and an exaggerated natriuretic response to saline infusion. This hypothesis proposes that the kidney, which has been shown to be consistently implicated in the pathogenesis of genetic hypertension (for a review see de Wardener, 1990a and 1990b), has two populations of nephrons:

(a) ischaemic nephrons, which produce excessive renin and result in increased angiotensin II concentrations and hence sodium retention;

(b) normal nephrons, which under the influence of the resultant increased angiotensin II concentrations produce less renin than normal.

Under these circumstances the extent to which net tubular sodium reabsorption occurs will depend on the balance between the effect of angiotensin II on ischaemic and normal tubules and the perfusion pressure affecting each nephron. When the pressure increases or there is a saline load the effects on the normal nephrons predominate and a natriuresis occurs.

Sealey et al. provide a great deal of evidence to support their hypothesis, and it is relevant to the hypothesis I will propose below.

10.24 The hypothesis that changes in Na^+/H^+ antiport activity are associated with vascular smooth muscle hypertrophy

The observations that Na^+/H^+ antiport activity increases after exposure to mitogenic stimuli and that Na^+/H^+ antiport activity is increased in hypertension have led to the hypothesis that vascular smooth muscle hypertrophy in hypertension is related to increased Na^+/H^+ exchange (Lever, 1986). This view is supported by the observation that both angiotensin II and noradrenaline increase the activity of the Na^+/H^+ antiport (Aalkjær and Cragoe, 1988; Berk et al., 1987a) and also cause vascular smooth muscle cell growth in culture (Campbell-Boswell and Robertson, 1981; Blaes and Boissel, 1983). This hypothesis is also relevant to the hypothesis I will propose below.

10.3 A NEW HYPOTHESIS

Any hypothesis which links abnormalities in ion transport with the pathophysiology of hypertension should explain why there are ion transport abnormalities in essential hypertension and should also explain the following observations:

Differences between plasma renin activities in different subgroups of hypertensive patients, who may have low, normal, or high plasma renin activity (Brunner et al., 1972), in association with differences in salt-sensitivity from patient to patient, which to some extent correlate with differences in renin (Laragh, 1973; Case et al., 1978).

The paradox that there is on the one hand increased sodium reabsorption from the proximal tubules of the kidney both in patients with essential hypertension (Weber, 1986) and in animals with genetic hypertension (Kato et al., 1986) and on the other a low or normal plasma volume and blood volume (Tarazi et al., 1968; Dustan et al., 1973) and an exaggerated natriuretic response to infusion of saline (Farnsworth, 1946; Cottier et al., 1958; Papper et al., 1960).

The development of abnormalities of ion transport before the development of hypertension (Henningsen et al., 1979; Woods et al., 1981; Neilsen et al., 1988).

The hypertrophy of vascular smooth muscle in hypertension, which is found in association with abnormalities of ion transport (Lever, 1986; Postnov, 1990).

The natural tendency of blood pressure to increase with advancing age (James et al., 1986).

This hypothesis proposes that the vascular hypertrophy and the abnormalities of ion transport which are found in essential hypertension are primarily due to patchy hypoperfusion of nephrons, which in turn leads to increased secretion of angiotensin II. This is as proposed by Sealey et al. (see above). Angiotensin II, in association with angiotensin II-induced increased sympathetic nervous activity (Mathias, 1987, Matsukawa et al., 1988; Fink et al., 1986; Webb et al., 1988), then causes both vascular smooth muscle hypertrophy and abnormalities of ion transport. This hypothesis is illustrated in outline in Fig. 10.1, and explains all of the above features of hypertension. The details of this hypothesis are shown in Figs. 10.2-4.

10.31 Alterations of ion transport systems in response to angiotensin II and/or noradrenaline

Fig. 10.2(a) and (b) shows the sequence of events that I propose occur after stimulation of vascular smooth muscle cells by angiotensin II.

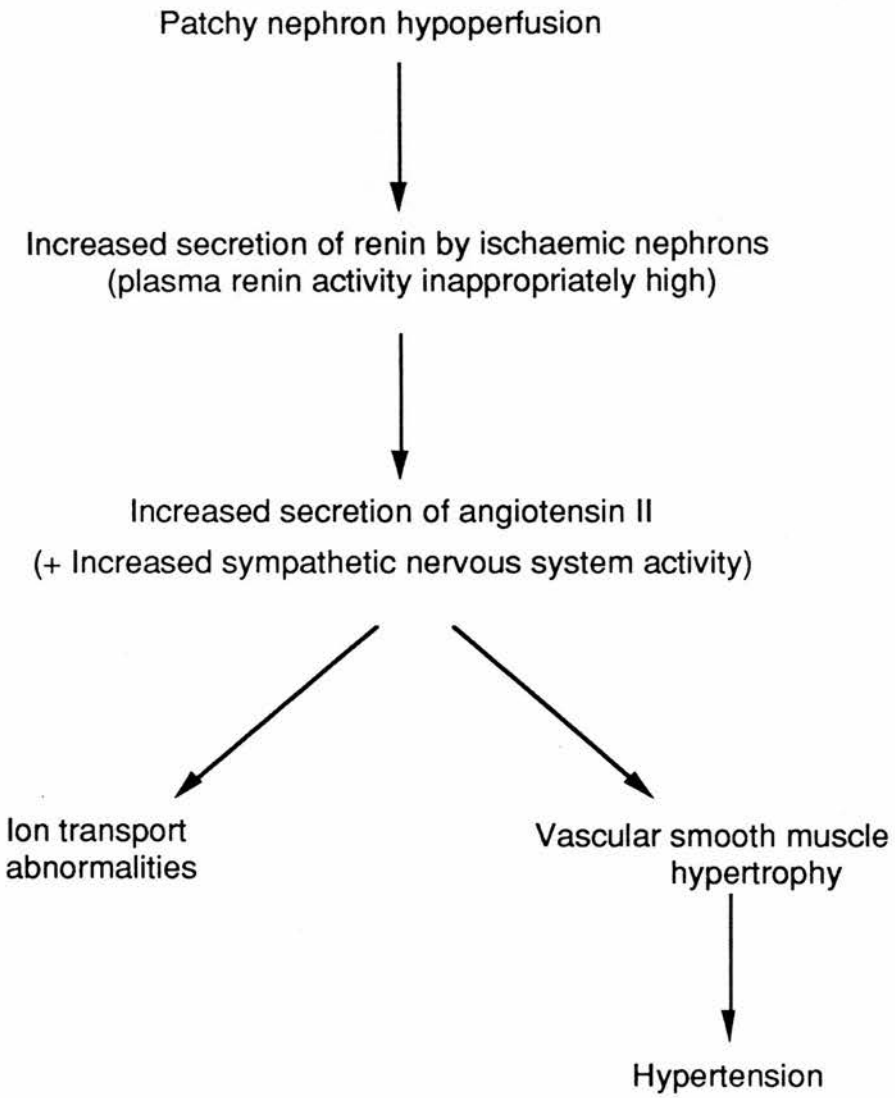

Fig. 10.1 Schematic diagram outlining the effects of partial renal ischaemia.

10.311 *Membrane depolarization and a tendency to increase cell volume*

The first effect of angiotensin II and also noradrenaline (as mentioned above) is membrane depolarization in association with activation of the inositol phosphate pathway and the opening of L-type calcium channels, resulting in increased influx of calcium (Berk et al., 1987b; Allen et al., 1988). In chapter 8 it was argued that this increase in calcium influx would lead to a local increase in calcium concentration at the cell membrane (Carafoli, 1988) without necessarily increasing the cytosolic concentration of free calcium. This local increase in membrane calcium, in conjunction with activated protein kinase C (for a review see Grinstein et al., 1986) increases the activity of the Na^+/H^+ antiport by altering its affinity for H^+ ions and so efflux of H^+ ions out of the cell becomes more efficient. This increase in Na^+/H^+ antiporter activity is necessary for homoeostatic control of intracellular pH, since there is increased production of acid resulting from ATP hydrolysis when cells are stimulated to contract under the influence of angiotensin II or noradrenaline. This intracellular acidosis would reduce the effective intracellular concentration of bicarbonate, and result in an influx of bicarbonate via a sodium-dependent bicarbonate/chloride exchange (Cala and Grinstein, 1988). This is illustrated in Fig. 10.2a.

If this process continued unabated it would result in sustained membrane depolarization and an increase in the intracellular concentration of sodium and thus cell swelling. However, this does not occur, since other mechanisms come into play.

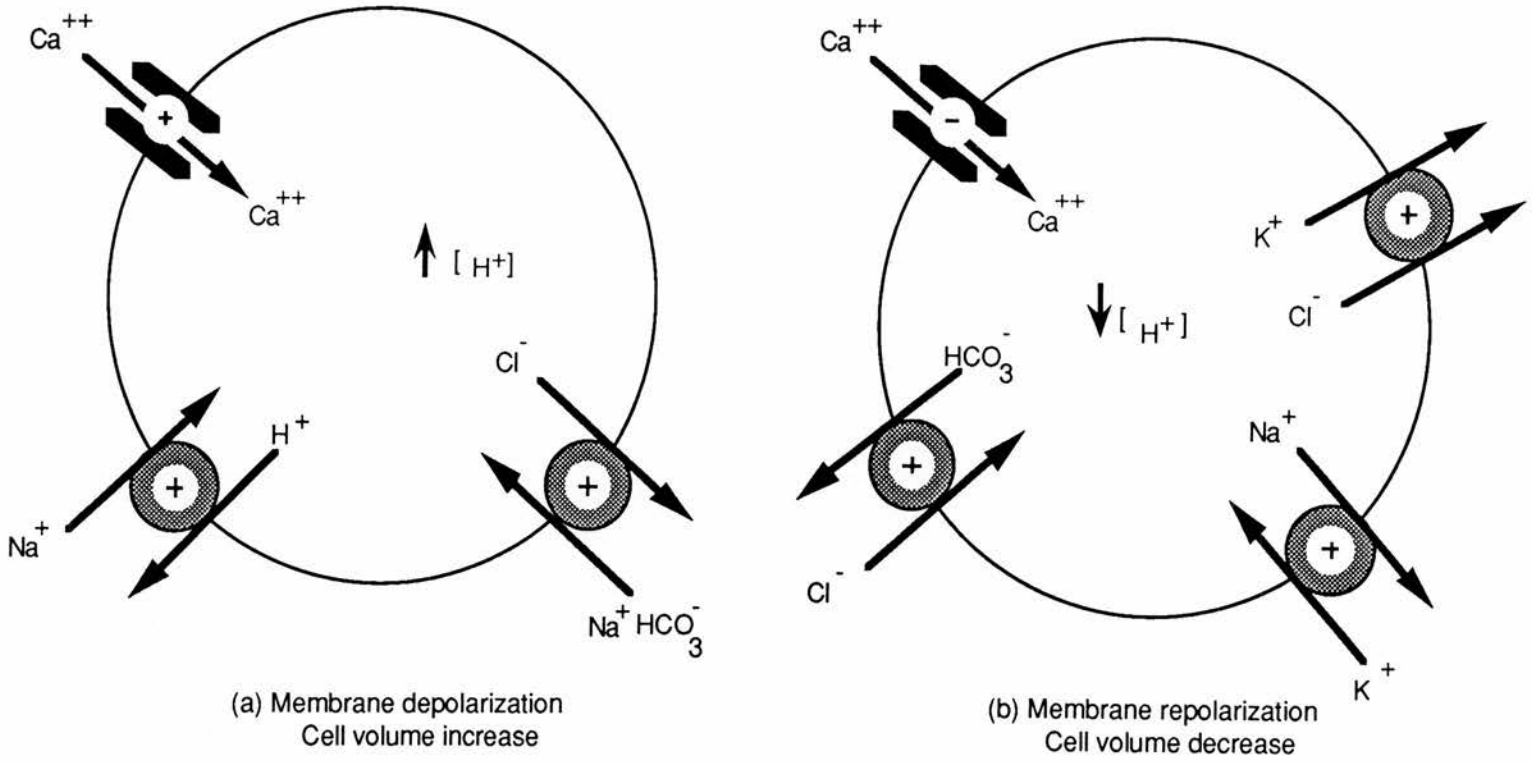

Fig. 10.2 Angiotensin II/noradrenaline-induced changes in ion fluxes in vascular smooth muscle cells resulting in (a) cell volume increase and (b) cell volume decrease.

10.312 *Membrane repolarization and a tendency to decrease cell volume*

In response to the tendency of the cell to swell, as described above, there will be a counter-regulatory decrease in cell volume due to increased potassium efflux, resulting from the opening of volume-sensitive potassium channels (for a review see Hoffman and Simonsen 1989). Since potassium efflux will be accompanied by chloride efflux via K^+/Cl^- co-transport, the intracellular chloride concentration would tend to fall. However, this would be opposed by chloride influx via a sodium-independent bicarbonate/chloride exchange (Cala and Grinstein, 1988), which would also oppose the bicarbonate influx described above. This is illustrated in Fig. 10.2b.

The net effect of this combination of ion movements across the cell membrane would be an increase in the intracellular sodium concentration and a reduction in the intracellular potassium concentration. These changes would activate the Na^+/K^+-ATPase (for a review see Schwartz et al., 1975) (Fig. 10.2b), in turn restoring the concentrations of sodium and potassium to normal.

Thus, the multiple findings of increased fluxes of Na^+ ions and K^+ ions in essential hypertension, in association with normal intracellular concentrations of sodium and potassium, can be explained by a sequence of linkage of different ion transporters which are involved in the regulation of pH, cell volume, and intracellular ion concentrations, all resulting from a physiological response of the cell to stimulation by agonists

such as angiotensin II and noradrenaline. These events are consistent with the *in-vivo* ion transport abnormalities discussed above in essential hypertension (Boon et al., 1986) and also those presented in earlier chapters of this thesis namely increased Na^+/H^+ antiport activity in the SHR (see Chapters 5 and 6) , which can be removed by blocking calcium influx via L-type calcium channels (see Chapter 8), increased potassium (rubidium) influx via the Na^+/K^+-ATPase (see Chapter 4), increased potassium (rubidium) efflux in SHRs (see Chapter 4), the same intracellular steady-state concentration of potassium (rubidium) despite increased influx and efflux in SHRs (see Chapter 4), and the same steady-state concentration of intracellular hydrogen ions and bicarbonate in the SHR (see Chapter 5 and 6). Finally, because it has been suggested that altered Na^+/H^+ antiporter activity is linked to altered Na^+/Li^+ countertransport in essential hypertension (Canessa, 1988), increased agonist stimulation of the cell resulting in this sequence could also explain the finding of increased Na^+/Li^+ countertransport in patients with essential hypertension *in vivo* (Brearley et al., 1990; Brearley et al., 1990).

At this juncture two points should be stressed. First, that despite these increases in the activities of numerous ion transport systems in hypertension, steady-state intracellular concentrations of potassium (rubidium) (see Chapter 4), hydrogen ions (see Chapters 5 and 6) and bicarbonate (see Chapter 6) are not different, since the various changes in fluxes tend to oppose one another. Second, that these changes in ion transport would *precede* the development of clinical hypertension, which I believe is more likely to be dependent on vascular smooth

muscle remodelling and hypertrophy, as others have previously suggested (Folkow et al., 1973; Folkow et al., 1982; Lever, 1986; Struyker Boudier et al., 1990). This is consistent with the observation that the normotensive first-degree relatives of patients with essential hypertension, who are at risk of developing hypertension in later years, have the same ion transport abnormalities (Henningsen et al., 1979; Woods et al., 1981; Neilsen et al., 1988).

In the next section I propose that vascular smooth muscle hypertrophy occurs in response to *long-term* agonist stimulation, in contrast to the changes in ion transport, which can occur more quickly. However, it is likely that the two processes are closely linked.

10.32 Vascular smooth muscle hypertrophy in response to angiotensin II and noradrenaline

I propose that vascular smooth muscle hypertrophy in hypertension results from a gradual accumulation of calcium in the sarcoplasmic reticulum and endoplasmic reticulum of the vascular smooth muscle cell over a number of years, due to a prolonged increase in agonist activity by for example angiotensin II and also noradrenaline. The proposed mechanism for this effect is illustrated in Fig. 10.3.

Fig. 10.3 shows the changes in calcium disposition which I propose are responsible for gradual hypertrophy of vascular smooth muscle in hypertension. There is reduced efflux of calcium because of reduced

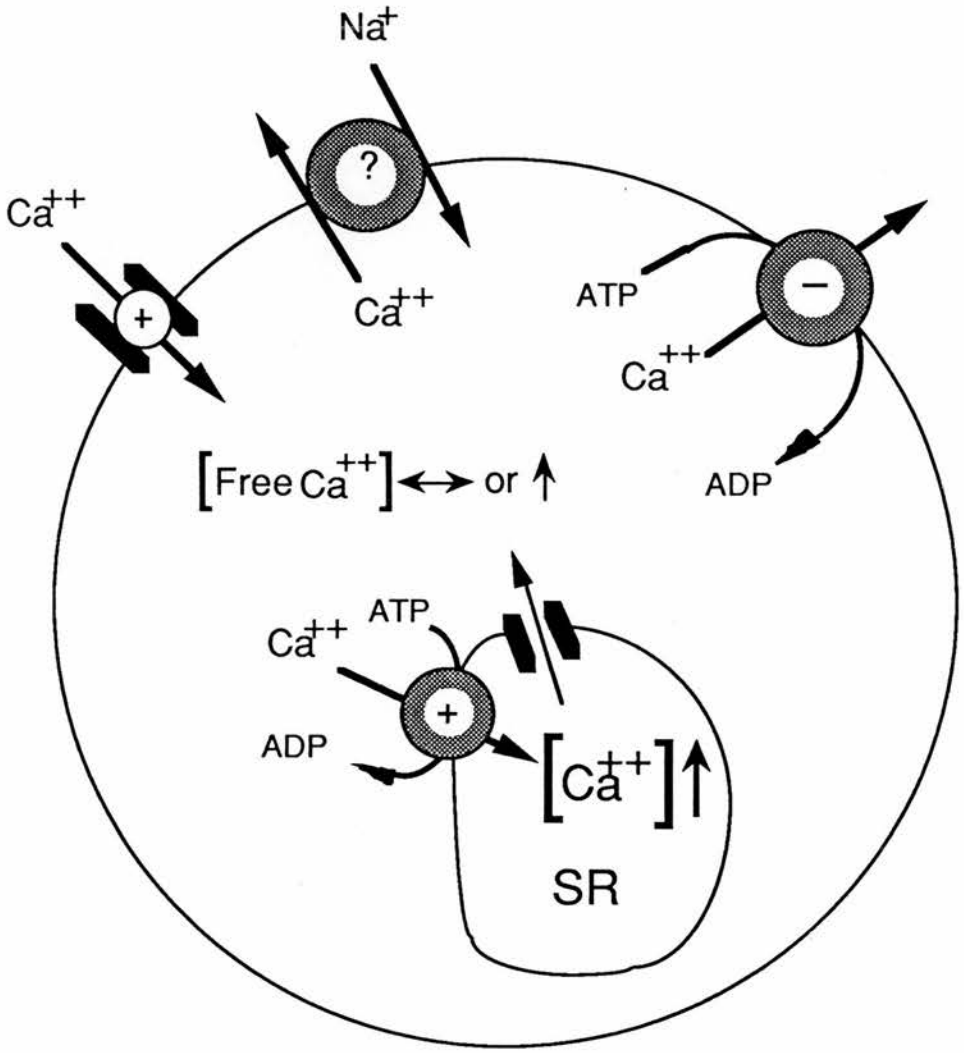

Fig. 10.3 A diagram of the changes in vascular smooth muscle cell handling of calcium resulting from chronic angiotensin II/noradrenaline stimulation.

activity of sarcolemmal Ca^{++}-ATPase (for reviews see Postnov and Orlov, 1985 and Dominiczak and Bohr, 1990); and an increased rate of calcium influx (Rusch and Hermsmeyer, 1988; for reviews see Postnov and Orlov, 1985 and Dominiczak and Bohr, 1990). Both of these fluxes occur via the plasma membrane. If these two alterations in fluxes continued unopposed the cytosolic calcium concentration would rise. However, increased flux of calcium from the cytosol into the sarcoplasmic reticulum (for a review see Carafoli, 1987) would tend to oppose an increase in the cytosolic concentration of calcium, keeping it at or near normal, while sarcoplasmic reticular calcium (and hence sequestered cell calcium) would increase. Reports of increased sequestered cell calcium in hypertension are consistent with this (Tobian and Chesley, 1966; Postnov and Orlov, 1985; Speiker et al., 1988).

Increased accumulation of calcium in the sarcoplasmic reticulum and endoplasmic reticulum of vascular smooth muscle will then increase protein synthesis (Brostrom and Brostrom, 1990) and cause cellular hyperplasia (Geering, 1986). Thus, this gradual accumulation of calcium will result in vascular remodelling and hypertrophy, establishing increased peripheral resistance and increased blood pressure.
There is evidence which supports the above sequence of events. Whereas it has been shown that *acute* stimulation of the vascular smooth muscle cells with angiotensin II and nordrenaline results in net efflux of calcium and a *reduction* in total cell calcium (Berk et al., 1987b; Hatori et al., 1987; for a review see Exton, 1985), there is evidence that *prolonged* stimulation with both angiotensin II and

noradrenaline produces net influx of calcium and an *increase* in the total cell calcium (Prpic et al., 1984; Joseph et al., 1985; for reviews see Exton, 1985 and Exton, 1988). This results because prolonged stimulation with both angiotensin II and noradrenaline *increases* calcium influx into the cell via increased opening of L-type calcium channels (Allen et al., 1988; Exton, 1988) and *reduces* calcium efflux by reducing the activity of sarcolemmal Ca^{++}-ATPase (Prpic et al., 1984; Exton, 1988).

It should be noted that both increased calcium influx via L-type channels and decreased activity of sarcolemmal Ca^{++}-ATPase are consistent findings in hypertension which have to date been explained by an intrinsic membrane abnormality (see above). However, it is clear that an alternative explanation for these findings is chronic agonist stimulation of the vascular smooth muscle cell by angiotensin II and noradrenaline.

10.33 Reasons for altered renal sodium handling in hypertension

As outlined above the hypothesis of Sealey et al.(1988) states that the kidney in hypertension has two populations of nephrons, hypoperfused nephrons, which produce excessive renin and result in increased angiotensin II concentrations and hence sodium retention, and normal nephrons, which under the influence of the resultant increase in angiotensin II concentrations produce less renin than normal. Whether or not there is a tendency towards sodium retention and volume expansion

then depends on the balance between sodium retention by hypoperfused nephrons and sodium loss by normal nephrons.

Based on this hypothesis and in conjunction with my own suggestion that long-term stimulation by angiotensin II and/or noradrenaline will increase sequestered cell calcium, I propose a sequence of events which I believe provides an explanation for the apparent paradox that salt retention (Weber, 1986; Kato et al., 1986) co-exists in hypertension with an exaggerated natriuretic response to infusion of saline (Farnsworth, 1946; Cottier et al., 1958; Papper et al., 1960). A schematic representation of the proposed sequence is shown in Figures 10.4a and b.

Increased circulating concentrations of angiotensin II will both increase sodium reabsorption in the proximal renal tubule (Harris and Navar, 1985; Schuster, 1986) and cause increased release of aldosterone (Quin and Williams, 1988). Both of these effects will cause sodium retention in all nephrons, whether hypoperfused or not (Figure 4a). This would lead to volume expansion which would however be opposed by the secretion of atrial natriuretic factor (ANF) (for a review see Ballermann and Brenner, 1985) and possibly also the putative inhibitor of Na^+/K^+-ATPase (for reviews see de Wardener and Clarkson 1985 and Poston, 1987). It should be noted that these actions of angiotensin II in causing sodium retention are both short-term and long-term actions.

In parallel with these effects, I believe (Figures 10.3 and 10.4b) that angiotensin II and noradrenaline increases sequestered intracellular calcium concentrations and that this results not only in vascular smooth muscle hypertrophy (as described above) but also in a further reduction in the secretion of renin in normal glomeruli through accumulation of calcium in the juxtaglomerular cells (for a review see Fray et al., 1987). This combination of increased pressure and reduced renin from normal glomeruli results in increased sodium loss from the normal nephrons (i.e. a pressure natriuresis). This in turn leads to volume reduction, which will switch off the secretion of ANF and the putative inhibitor of Na^+/K^+-ATPase. This effect would also explain the saline-induced natriuresis of hypertension, because saline load would cause a further increase in the sodium load in the nephron. Nevertheless, hypoperfused nephrons will at the same time continue to drive the production of angiotensin II (Figure 10.4a), whose production will not be switched off. Thus, the overall balance between the effects of hypoperfused nephrons and the effect of normal nephrons will determine sodium and volume homoeostasis in hypertension.

10.34 Reasons for different renin subgroups and degrees of salt sensitivity in essential hypertension

As the secretion of renin and the formation of angiotensin II should be completely inhibited with an increase in renal perfusion pressure (Tobian, 1960; Vander, 1967) it follows that in established hypertension even when the plasma renin activity is *low* there is inappropriately *high* renin secretion. Thus, in all forms of hypertension there is de-

tectable activity of renin in the plasma because of nephron hypoperfusion. I therefore propose that the different plasma renin activities in hypertension relate to different grades of nephron hypoperfusion. From this one would predict that in hypertension with low renin activity the blood pressure would be more dependent on volume expansion (i.e. relatively more salt-sensitive) than it is on renin and angiotensin II, while in hypertension with high renin activity the blood pressure would be more dependent on renin and angiotensin II (i.e. relatively less salt-sensitive). This is a recognized association (Laragh, 1973; Case et al., 1978).

With an increasing degree of nephron hypoperfusion and increased renin production one might expect increased sodium reabsorption and volume expansion (Figure 4a). However, I propose that this does not happen, because the increased blood pressure increases the perfusion pressure of normal glomeruli and increases natriuresis (Figure 10.4b), which therefore predominates over sodium retention.

With worsening ischaemia there is further sodium loss and plasma volume reduction, which in turn causes a further reduction in blood flow to the kidney, leading to a further increase in renin and a upward spiral of increasing blood pressure. I believe that this happens in the extreme case of malignant hypertension, in which very high concentrations of renin are associated with sodium loss and plasma volume reduction (for a review see Houston, 1989).

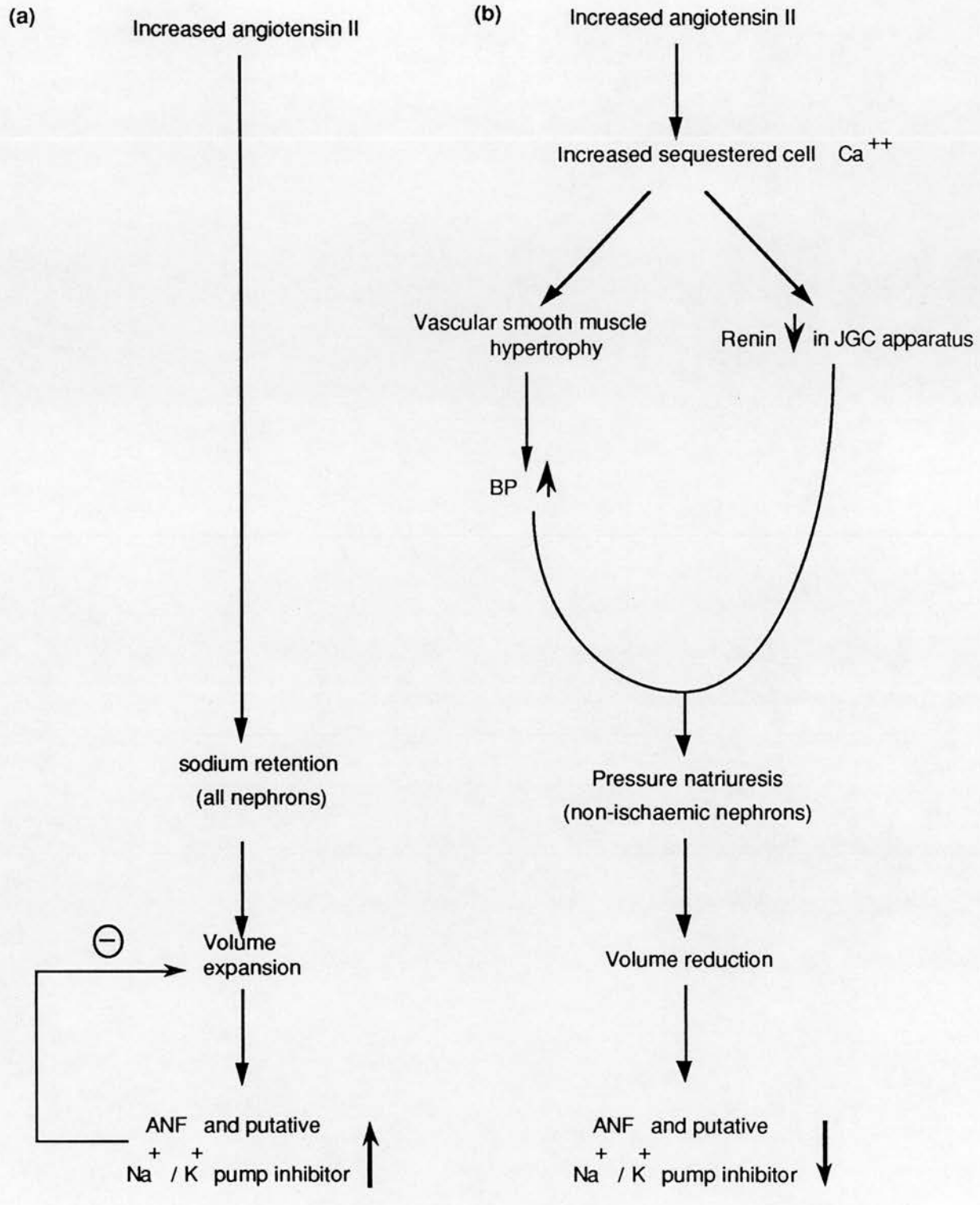

Fig. 10.4 A schematic outline of (a) the sodium-retaining effects of increased angiotensin II stimulation and (b) the pressure natriuresis effect of prolonged angiotensin II stimulation.

10.35 Changes in blood pressure with age

Increases in total calcium have been reported to occur with increasing age, and this may explain the findings of vascular smooth muscle hypertrophy and the development of increased blood pressure with increasing age (James et al., 1986), as discussed above. This could result from angiotensin II and/or noradrenaline stimulation over many years. These effects would also explain the observation of a reduction in plasma renin activity with increasing age (for a review see Swales, 1989), since this would result from a combination of a reduced number of nephrons and the renin-lowering effects of an increased total intracellular calcium (Fray et al., 1987). One would also predict that blood pressure in the elderly would be relatively sensitive to the effects of salt.

10.4 SUMMARY AND PREDICTIONS

I have outlined above the observations which I believe should be explained by a hypothesis which links abnormalities in ion transport with the pathophysiology of hypertension. The above hypothesis fulfils this criterion, since it provides an explanation for the ion transport abnormalities found in genetic hypertension in animals and in man and in addition it explains:

1. The differences between plasma renin activities in different subgroups of hypertensive patients in association with differences in salt-sensitivity from patient to patient.

2. The paradox that there is on the one hand increased sodium reabsorption from the proximal tubules of the kidney in hypertension and on the other hand low or normal plasma and blood volumes and an exaggerated natriuretic response to infusion of saline and to increased pressure.

3. The development of abnormalities of ion transport before the development of hypertension.

4. The hypertrophy of vascular smooth muscle in hypertension.

5. The tendency for blood pressure to increase with advancing age.

The multiple ion transport abnormalities found in genetic hypertension can be explained by chronic stimulation by agonists such as angiotensin II and noradrenaline, without the need to propose that an abnormal cell membrane is responsible. Furthermore, as discussed above, the changes in the composition of the membrane could be caused by these ion transport abnormalities.

Certain predictions arise from this hypothesis. For example, it is likely that different ion transport abnormalities and volume status will be found at different times during the development of hypertension. One would also predict a change in the number of both angiotensin II receptors and possibly α_1-adrenoceptors with the development of hypertension, in keeping with the changes that would be expected from

chronic stimulation of these receptors (Colucci and Alexander, 1986; for a review see Exton, 1988). However, the *negative* effect this would have on vascular responsiveness would have to be balanced against the *positive* effect of angonist-induced calcium loading of the vascular smooth muscle cell. It is also clear that certain drugs would be more likely to be effective in certain sub-types of hypertension. Finally, if this hypothesis is correct essential hypertension is a potentially reversible condition which results in premature ageing of the vascular system.

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